Fermentation strategies for sorghum grain applications

by

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Abstract

Sorghum is a cereal grain with significant potential for food applications due to its composition, gluten-free nature, and high antioxidant content. However, its use remains limited by intrinsic factors such as condensed tannins, phytic acid, and enzyme inhibitors, which can reduce nutrient availability and functionality. Fermentation represents a promising strategy to modify these properties and enhance sorghum value through microbially driven transformations.

The objective of this research was to evaluate fermentation strategies to expand the use of sorghum grains using both submerged and solid-state systems, and to determine how sorghum variety and fermentation conditions influence microbial dynamics. To address this objective, three complementary studies were conducted.

In the first study, three whole sorghum grain varieties, sumac, waxy, and white, were used as substrates for kombucha fermentation and evaluated over 7 days by monitoring pH, soluble solids (°Brix), microbial counts, and microbial community composition using shotgun metagenomic sequencing. All treatments exhibited a significant decrease ($p<0.05$) in pH and °Brix, along with an increase in microbial population, indicating active fermentation. Metagenomic analysis showed that sorghum-based fermentations maintained microbial profiles similar to those of the starter culture, indicating that sorghum can support kombucha fermentation while preserving its characteristic microbiota. To further assess potential functional effects, the second study evaluated the effects of sorghum kombucha consumption in an obesity intervention model using Ossabaw pigs. Animals received either sorghum kombucha or an isocaloric tea control, and fecal samples were analyzed for *Enterobacteriaceae* counts and microbial community composition. Kombucha supplementation significantly reduced *Enterobacteriaceae* shedding ($p < 0.05$) across body types. However, beta diversity analysis

indicated that host body type had a greater influence on microbial community composition than treatment, highlighting the importance of host-related factors in shaping microbiome responses. In the third study, lactic acid bacteria-mediated solid-state fermentation was used to ferment three whole sorghum grain varieties (i.e., black, sumac, and white), under different moisture conditions and inoculum treatments. Fermentation resulted in significant reductions in phytic acid and pH ($p < 0.05$), along with increases in microbial populations. In vitro protein digestibility improved significantly in selected treatments, particularly in black and sumac sorghum varieties. The observed effects were influenced by sorghum variety, inoculum type, and moisture level.

Collectively, these findings demonstrate that fermentation can be an effective strategy to enhance sorghum functionality and broaden its applications. Sorghum supported fermentation in both submerged and solid-state systems. However, fermentation dynamics were strongly influenced by substrate characteristics and processing conditions, highlighting the need for tailored fermentation approaches. These findings provide a foundation for developing targeted strategies and support the use of sorghum as a versatile ingredient in functional food applications.

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Dedication

I dedicate this thesis to my family. Specifically, to Mom, Virna, thanks for teaching me to be resilient and for always encouraging me to give my very best.

Chapter 1 - Literature Review

Fermentation and its role in enhancing the nutritional value of foods

Fermentation is one of the oldest food processing techniques, practiced since the early development of human civilizations (Marsh et al., 2014). While traditionally used to preserve food and extend shelf life, fermentation is now widely recognized for its contributions to food safety, nutritional enhancement, and improved sensory properties (Ansorena & Astiasarán, 2016). Fermentation is a microbially driven process in which complex substrates, such as carbohydrates and proteins, are broken down into simpler compounds through microbial enzymatic activities. Beyond substrate degradation, microorganisms involved in fermentation contribute to the release and production of metabolites, including phenolic derivatives, peptides, and short-chain fatty acids (SCFAs) (Ghosh et al., 2013). These compounds have been associated with a variety of health benefits, such as antioxidant, anti-inflammatory, antidiabetic, and cardioprotective effects (Limón et al., 2015; Yadav et al., 2012).

Phenolic compounds and peptides are two classes of bioactive compounds known for their significant health-promoting properties. Phenolic compounds, including phenolic acids, flavonoids, and anthocyanins, are widely recognized for their antioxidant properties (Hur et al., 2014; L. Liu et al., 2024), as well as their anti-inflammatory, antidiabetic, and cardioprotective effects (Limón et al., 2015; Yadav et al., 2012). Similarly, bioactive peptides, which are short amino acid sequences embedded within larger protein structures that become active upon release (Sánchez & Vázquez, 2017), have been linked to a broad spectrum of health-promoting functions, including immune modulation, antioxidant activity, angiotensin-converting enzyme (ACE) inhibition, and antimicrobial properties (Admassu et al., 2018; Venegas-Ortega et al., 2019).

During fermentation, the levels of both phenolic compounds and bioactive peptides can significantly increase. This enhancement is primarily due to microbial enzymatic transformations, as microorganisms possess enzymes that convert bound and conjugated forms of phenolic compounds into bioavailable structures (Gänzle, 2014). Additionally, microbial proteases can degrade larger food proteins into smaller, bioactive peptide fragments (Cruz-Casas et al., 2021). As these proteins are broken down into smaller peptides, their bioavailability and biological activity are enhanced. Salar et al., (2012) reported a positive correlation between the activity of carbohydrate-degrading enzymes (such as α-amylase, β-glucosidase, and xylanase) and increased total phenolic content and antioxidant activity during solid-state fermentation of maize with the filamentous fungus *Thamnidium elegans* after 6 days of fermentation. Similarly, Hole et al., (2012) observed enhanced bioavailability of phenolic compounds, specifically an increase in free phenolic acids, in whole grain barley and oats flour fermented with *Lactobacillus acidophilus, Lactobacillus johnsonii,* and other *Lactobacillus* species. Elfahri et al., (2016) observed that fermenting milk with *Lactobacillus helveticus* strains and their crude protease extracts released bioactive peptides with ACE-inhibitory, antioxidant, and immunomodulatory properties. Similarly, Rizzello et al., (2017) reported the production of short peptides with strong antioxidant activity after 24 hours of fermenting quinoa flour with *Lactobacillus plantarum*. These peptides not only remained effective after simulated digestion but also protected human cells from oxidative stress, further highlighting the potential of fermentation to increase beneficial health effects of food products.

Another advantage of fermentation is the presence of probiotic culture. Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit to the host (WHO, 2001). These beneficial microbes play a critical role in modulating the composition and function of the intestinal microbiota. The key mechanism is the enhanced

production of SCFAs, primarily acetic, propionic, and butyric acids (S. Li et al., 2023; Sheng et al., 2021). These SCFAs are crucial mediators of gut homeostasis and overall host health. Butyrate, for instance, is the primary energy source for colonocytes, essential for preserving the integrity and function of the intestinal epithelial barrier (Kopczyńska & Kowalczyk, 2024). Propionate diffuses into the hepatic portal vein, where it is utilized for hepatic gluconeogenesis and can also influence satiety and blood lipid levels (Hays et al., 2024; Pant et al., 2023). Beyond these direct metabolic benefits for gut and liver, SCFAs also act as vital signaling molecules, influencing systemic immune responses and broader metabolic functions throughout the body (Hays et al., 2024).

Fermentation also plays a crucial role in reducing antinutritional factors (ANFs) present in many raw food materials. Compounds such as phytic acid, tannins, and trypsin inhibitors are known to interfere with nutrient absorption (Salim et al., 2023; Salim-ur-Rehman et al., 2014). By decreasing the levels of these compounds, fermentation improves macronutrient digestibility and enhances the overall nutritional profile of fermented products, especially in legumes and cereal-based products (Jeyakumar & Lawrence, 2022). Lactic acid bacteria have demonstrated a notable effect in mitigating these antinutritional compounds. A study by Adeyemo & Onilude, (2013) evaluated *Lactobacillus plantarum* strains during the 5-day fermentation of soybean blends and reported the hydrolysis of soybeans and a reduction of antinutritional factors, including tannin, phytate, trypsin inhibitor, and protease inhibitor. Similarly, Fang et al., (2023) observed significant phytase activity and a high degradation of phytic acid during sourdough fermentation involving a mixed microbial culture, including *L. paracasei, L. plantarum, L. lactis,* and *Saccharomyces cerevisiae* strains. Terefe et al., (2021) further evaluated the effects of *L. plantarum* and *S. cerevisiae* individual and co-cultures on maize flour during a 48-hour fermentation. While their

findings showed the highest in vitro protein digestibility with *L. plantarum* alone, the study crucially reported significant reductions in phytate, tannins, and trypsin inhibitors, with the co-culture demonstrating the most pronounced decrease in antinutritional factors.

Overall, all these findings underscore the potential of fermentation as an effective strategy for enhancing the nutritional and functional qualities of food, highlighting its relevance in the development of health-oriented food products through the release and production of metabolites with potential antioxidant, anti-inflammatory, antidiabetic, and cardioprotective properties and effects.

Types of Fermentation: Solid-State vs Submerged

Fermentation processes are generally categorized into solid-state fermentation (SSF) and submerged fermentation (SmF), based primarily on the physical state of the substrate and the environmental conditions that support microbial growth (X. Liu & Kokare, 2017). These differences significantly influence microbial ecology, metabolic activities, and the resulting biochemical transformations, thereby shaping their use in various food and industrial applications (Chen, 2013).

Solid state fermentation (SSF)

SSF is a process where microorganisms grow on solid substrates with little to no free-flowing water (Khosravi & Razavi, 2021). This system has been used for centuries, especially in East Asian cultures, to produce fermented foods like soy sauce, miso, and koji (Barrios-González, 2012). In recent decades, SSF has gained renewed interest due to its versatility and sustainability, with applications expanding across various industries. It is now commonly used for producing high-value compounds such as enzymes, organic acids, secondary metabolites, biofuels, and biopesticides (Lizardi-Jiménez & Hernández-Martínez, 2017). These various

applications are largely enabled by the wide range of microorganisms that can grow in SSF conditions, including fungi, bacteria, and yeasts (El-Naggar, 2009; Lizardi-Jiménez & Hernández-Martínez, 2017; Torino et al., 2013).

SSF operates as a three-phase system consisting of solid, liquid, and gas phases (Lizardi-Jiménez & Hernández-Martínez, 2017). The solid matrix, which acts as both support and nutrient source, can be classified as either a nutritional or an inert carrier. Nutritional carriers usually provide essential carbon and nitrogen sources (Sadh et al., 2018; Singhania et al., 2010a; Zhang et al., 2017), while inert supports are impregnated with a nutrient solution to facilitate microbial colonization (Osmolovskiy et al., 2021; Thomas et al., 2013; X. Xu et al., 2011).

Moisture content, typically ranging from 12% to 80%, with most processes operating around 60%, is a critical parameter in SSF since it directly influences microbial growth and metabolic activities (Chen, 2013): excessive moisture can reduce substrate porosity and oxygen transfer, while insufficient moisture may hinder nutrient diffusion and microbial metabolism (Yang et al., 2021). The gas phase also plays a crucial role, particularly in providing oxygen supply and facilitating carbon dioxide (CO_2) removal, is essential for aerobic microbial respiration (Oiza et al., 2022). Its composition and dynamics are influenced by substrate porosity, aeration strategy, and reactor's design (Chilakamarry et al., 2022).

Various bioreactor designs have been developed to address the unique challenges of SSF, particularly in optimizing heat and mass transfer (Table 1). Tray bioreactors are widely used for traditional food fermentations like tempeh and miso; they rely on passive aeration and are typically operated in temperature- and humidity-controlled rooms (Zhu & Tramper, 2013). Packed-bed bioreactors are tubular vessels packed with solid particles that provide support for microorganisms or substrates. These particles remain stationary throughout the process, as

mixing is generally avoided to protect microbial growth. Aeration is typically supplied through a perforated bottom to ensure adequate oxygen transfer. Such reactors are especially suited for processes where agitation could harm the microorganisms or disrupt the system (Arora et al., 2018a). Drum bioreactors introduce mechanical mixing, either through stirred-drum mechanisms, where internal paddles rotate within a stationary drum, or by rotating-drum systems, where the entire vessel rotates. Both enhance the distribution of heat, moisture, and oxygen throughout the solid substrate (Krishania et al., 2018; Mattedi et al., 2023b).

Overall, SFF offers advantages such as reduced water and energy requirements, lower operational costs, and simplified downstream processing (Gómez-Ramos et al., 2025; Karimi et al., 2021). Moreover, it allows for the use of agro-industrial residues as substrates to produce value-added metabolites at low cost (Sadh et al., 2018). However, SSF poses challenges, such as difficulties in monitoring and controlling pH, oxygen, and temperature. Furthermore, scaling up from laboratory to industrial applications remains challenging due to heat and mass transfer limitations, which can lead to uneven microbial growth and product formation (Mitchell et al., 2011; Pandey, 2003). Addressing these limitations requires careful reactor design, substrate selection, and process optimization strategies tailored to specific microbial starters used and target products.

Submerged fermentation (SmF)

SmF involves the cultivation of microorganisms in a liquid medium where substrates are fully dissolved or suspended in water (Ouedraogo & Tsang, 2021). Unlike SSF, SmF systems consist of a continuous aqueous phase, typically containing more than 95% water by weight (Chen et al., 2011). This homogenous system allows for efficient nutrient uptake and supports rapid microbial proliferation, especially for unicellular organisms such as bacteria (Doriya et al.,

2016). SmF provides a well-controlled environment where parameters such as pH, temperature, dissolved oxygen (DO), and agitation speed can be continuously monitored and adjusted through automated systems, enabling optimized conditions throughout the fermentation cycle (Adamian et al., 2021; R. Liu et al., 2019). It is widely used in the industry, from the production of foods, enzymes, antibiotics and bioactive compounds.

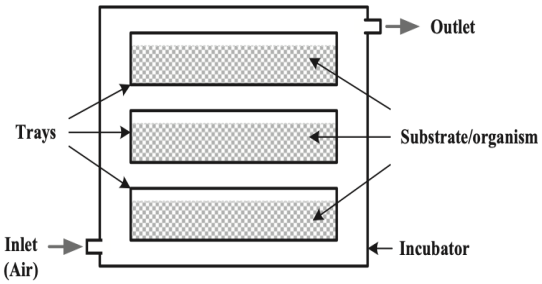
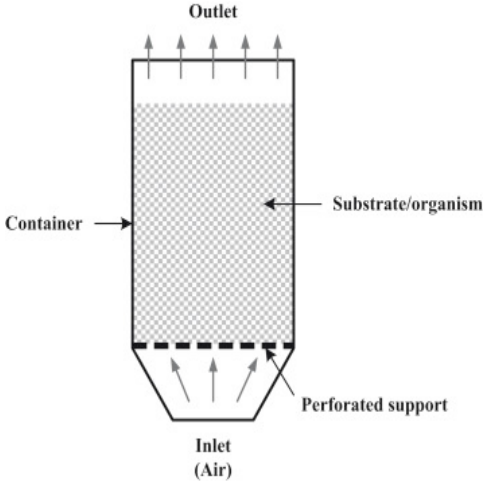
SmF can operate in batch, fed batch, or continuous modes. In batch fermentation, all nutrients are added at the beginning of the process, and the system remains closed until fermentation is complete (Ouedraogo & Tsang, 2021). Fed-Batch fermentation begins under batch conditions but involves the incremental addition of nutrients throughout the process, to prolong the growth phase and avoid substrate inhibition (S.-Y. Li et al., 2011). Continuous fermentation maintains steady-state conditions through the process, supporting consistent microbial activity and product formation (Yi, 2022) over extended periods of time (Zeng & Sun, 2014).

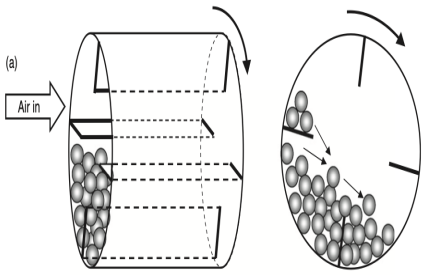
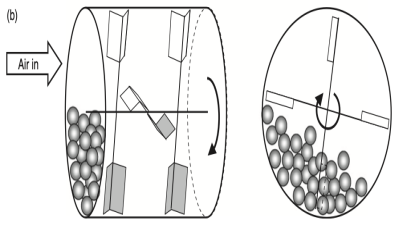
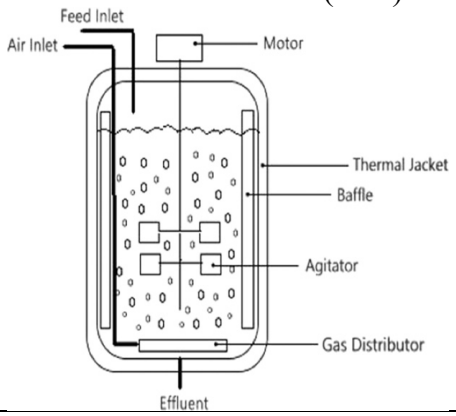
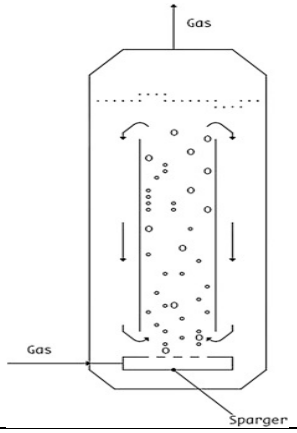
Different types of bioreactors are used in SmF (Table 1), with stirred-tank reactors (STRs) the most common. These systems are equipped with mechanical agitators and spargers to facilitate oxygen transfer and ensure uniform mixing (Bakratsas et al., 2021; Prado Barragán et al., 2016). For shear-sensitive organisms, alternatives such as airlift or bubble column reactors offer gentler mixing through air circulation rather than mechanical stirring (Palladino et al., 2024). Airlift bioreactors are vertical vessels designed for large-scale aerobic processes, where gas is introduced at the base to circulate and mix the culture medium via airlift-driven flow (Prado Barragán et al., 2016). Bubble column reactors are cylindrical vessels where gas is introduced at the bottom, forming bubbles that rise through the liquid to promote mixing and gas exchange (Prado Barragán et al., 2016). These systems support various industrial applications, including the production of

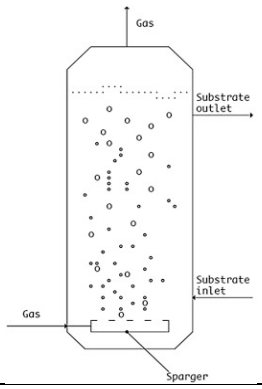
antibiotics, organic acids, biofuels, enzymes, and fermented foods and beverages such as kefir, beer, wine, and kombucha (Rivas et al., 2008; Singhania et al., 2010b).

SmF presents several advantages, such as scalability, reproducibility, ease of monitoring, and suitability for automated control and continuous operation (Adamian et al., 2021). However, the system also presents limitations such as high water and energy demands for sterilization, agitation, and aeration, which contribute to elevated operational costs. Foaming is commonly formed and may require antifoaming agents that interfere with oxygen transfer. Additionally, downstream processing is often more complex due to the dilute nature of the broth, and the larger volumes of wastewater generated (Jain et al., 2013; Krishna, 2008).

Table 1 Types of Fermentation Reactors

Type of Fermentation	**Bioreactor**	**Characteristics**	**References**
SSF	Tray Bioreactor 	Stack trays with spacing between layers, open tops and perforated bottoms, passive aeration.	(Arora et al., 2018b; Ge et al., 2017)
	Packed bed bioreactors 	Vertical cylindrical tube, substrate held by a plate, aeration from the bottom, temperature regulated by jackets or internal plates; periodic mixing and water spraying if needed.	(Chen, 2013; Ge et al., 2017)

	Rotating Drum Bioreactor 	Horizontal cylindrical drum partially filled with substrate, mixing by tumbling, aided by internal baffles, air blown into headspace for indirect aeration;	(Mitchel et al., 2011)
	Stirred Drum Bioreactor 	Stationary drum with internal agitator/paddles, air introduced into headspace for indirect aeration;	(Mitchel et al., 2011)
SmF	Stirred-Tank Reactor (STR) 	Most common SmF system; vertical vessel with axial or radial impeller for mixing, aeration, and heat/mass transfer	(Bakratsas et al., 2021)
	Airlift Reactor 	Tower reactor using compressed air for circulation and mixing, low shear stress; suitable for shear-sensitive and aerobic cultures.	(Prado Barragán et al., 2016)

	Bubble Column Reactor 	Cylindrical vessels, mixing via air bubbles; high heat and mass transfer; suitable for aerobic processes.	(Prado Barragán et al., 2016)

The Role and Importance of Starter Cultures

Microbial fermentation is defined as the biochemical process in which microorganisms metabolize food components, leading to the production of new compounds or the modification of existing ones (Sawant et al., 2025). The specific microbial species involved, their metabolic pathways, and the environmental conditions surrounding them influence these changes (Vinicius De Melo Pereira et al., 2020). Additionally, the microbial strategy employed, whether spontaneous or controlled fermentation, plays a key role in determining both the course and the outcome of the process (H. Li et al., 2020; Randazzo et al., 2017).

Spontaneous fermentation is a process driven by the naturally occurring microorganisms present on the substrate and/or the environment (Sionek et al., 2023; Skowron et al., 2022). The earliest fermented foods were produced through spontaneous fermentation, and today, many products like kimchi, cocoa, coffee, and wine are still made this way (Francesca et al., 2016; C. L. Schwan et al., 2023; Siddiqui et al., 2023). The microbial community composition in these fermentations is shaped by the interplay of substrate properties and environmental factors such as temperature, pH, and oxygen availability, as well as microbial interactions, competition, and succession (Mudoor Sooresh et al., 2023; Navarrete-Bolaños, 2012).

A well-documented example is cocoa fermentation. At the beginning of the process, the yeasts naturally present on the cocoa pulp dominate the microbial community, initiating sugar metabolism and producing ethanol, carbon dioxide, and heat, which lower pH. As ethanol and temperature levels increase, yeast populations decline, creating favorable conditions for LAB and later for AAB (Sarbu & Csutak, 2019). LAB further acidifies the environment, while AAB oxidizes ethanol into acetic acid. Together with rising temperature, these microbial activities diffuse into the cacao beans, kill the seed embryo, and trigger endogenous enzymatic reactions that generate flavor and color precursors (Moreira et al., 2022; R. F. Schwan & Wheals, 2004). The resulting biochemical transformations produce a wide variety of volatile and non-volatile compounds, including alcohols, aldehydes, esters, and pyrazines, which contribute to the characteristic aroma and quality of cocoa products (Rodriguez-Campos et al., 2011).

Although spontaneous fermentation promotes microbial diversity and unique sensory qualities (Medina et al., 2013), it also presents challenges, such as product variability and a higher risk of contamination (Holzapfel, 2002). To address these limitations, traditional practices such as backslopping (a semi-controlled method in which part of a successful fermentation is transferred to a new batch) were developed to improve consistency and favor desirable microorganisms (Kim et al., 2018; Liao et al., 2024). However, the expansion of large-scale industrial fermentation, which requires reproducibility, safety, and precise control of microbial physiology, highlighted the need for controlled fermentation and the development of starter cultures specifically adapted to industrial conditions (Navarrete-Bolaños, 2012; Vinicius De Melo Pereira et al., 2020).

Controlled fermentation is defined as the deliberate addition of starter cultures to direct microbial succession, thereby ensuring a predictable and consistent process (Voidarou et al.,

2020a). Starter cultures consist of one or more selected microorganisms intentionally added to the substrate to initiate and direct fermentation processes (Laranjo et al., 2019). These cultures are developed by isolating and selecting strains with desirable traits, enabling standardization of food production (Powell, 2025; Zarzecka et al., 2020).

Starter cultures provide several advantages for large-scale production, by rapidly dominating the microbial community, they inhibit spoilage organisms and potential pathogens through rapid acidification, nutrient competition, and the production of antimicrobial compounds such as bacteriocins (Francesca et al., 2016; Holzapfel, 2002). This reduces batch failures, contamination risks, and production losses (Corsetti et al., 2012; Hua et al., 2020). Moreover, strains are strategically selected for their enzymatic activities and metabolic byproducts to enhance nutritional quality, such as increasing bioactive compound levels or reducing anti-nutritional factors (Hwabejire et al., 2024; Mohammadi-Kouchesfahani & Hamidi-Esfahani, 2019; Romero-Espinoza et al., 2020). Because the metabolic profiles of starter strains are well characterized, they enable consistent batch-to-batch results and better control of flavor, texture, and aroma (Jia et al., 2021; Vinicius De Melo Pereira et al., 2020). This standardization is vital in industrial settings, where consistent outcomes are essential to meet consumer expectations (Galimberti et al., 2021; Sionek et al., 2023).

A practical example is cheese production, where known LAB are used as primary starters to ferment sugars into lactic acid, lowering pH and inhibiting spoilage and pathogenic microorganisms (Korena et al., 2023). Additional cultures, including non-starter LAB, AAB, yeasts, or molds, are often introduced to guide ripening and drive biochemical reactions that enhance sensory and physicochemical properties (Korena et al., 2023).

Types of Selected Starter Cultures

Selected starter cultures can be classified based on the number and diversity of microbial species they contain, as single-strain or mixed-strain cultures (Bintsis & Papademas, 2024). Single-strain cultures consist of a pure population of one well-characterized microorganism (Holzapfel, 2002). They are widely used in industrial processes where reproducibility and specific metabolic outputs are essential. Examples include *Saccharomyces cerevisiae* in beer production (Thesseling et al., 2019), and bread making (Parapouli et al., 2020), as well as particular lactic acid bacteria such as *Lactobacillus rhamnosus* or *Lactobacillus delbrueckii* ssp in the commercial production of lactic acid (Eş et al., 2018).

Mixed-strain cultures instead contain two or more microbial species or strains and can be further categorized into defined and undefined mixed cultures (Hesseltine, 1991). Defined mixed cultures are composed of well-characterized and quantified strains, such as the yogurt starter culture composed of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *Bulgaricus (Wasilewska et al., 2019).* Undefined mixed cultures, on the other hand, include microbial consortia whose overall composition is recognized but where individual strains are not fully identified or quantified (Powell, 2025). These are commonly associated with fermentations like kombucha or sourdough, where yeasts, lactic acid bacteria, and acetic acid bacteria interact to drive fermentation and produce distinctive sensory attributes (Harrison & Curtin, 2021; Siepmann et al., 2018). The choice between single-strain and mixed-strain cultures depends on the desired characteristics of the final product, the complexity of the flavor profile, and the specific requirements of the production process.

Common Microorganisms Used as Selected Starter Cultures

Selected starter cultures include a wide range of microorganisms, including LAB, AAB, yeasts, and fungi. Each of them follows characteristic metabolic pathways and enzymatic activities that mediate biochemical transformations within the food matrix (Marco et al., 2021; Voidarou et al., 2020b).

LAB comprises genera such as *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Pediococcus*, which are widely applied in food fermentations thriving in diverse food matrices, including dairy products, meats, vegetables, and bread (Bintsis & Papademas, 2024). Their primary role is the conversion of carbohydrates into lactic acid, leading to rapid acidification, inhibition of spoilage and pathogenic microorganisms, and development of flavor and texture (Mora-Villalobos et al., 2020). Based on their metabolic pathways and end products, LAB can be classified as homofermentative or heterofermentative (Settanni & Moschetti, 2010). Homofermentative LAB produce almost exclusively lactic acid via the Embden–Meyerhof–Parnas (EMP) pathway (Mozzi, 2016), whereas heterofermentative LAB use the pentose phosphate pathway to generate lactic acid along with acetic acid, ethanol, and carbon dioxide (Eiteman & Ramalingam, 2015).

AAB are aerobic microorganisms known for their ability to oxidize ethanol, carbohydrates, and sugar alcohols into acetic acid, other organic acids, aldehydes, or ketones (Lynch et al., 2019). AAB common genera include *Acetobacter, Komagateibacter* and *Gluconobacter*, which are applied in vinegar production and in fermented beverages such as kombucha and kefir (Gomes et al., 2018; Lynch et al., 2019; Xu et al., 2011).

Yeasts are essential microorganisms in the production of alcoholic beverages and leavened bakery products, where their anaerobic metabolism converts fermentable sugars into

ethanol and carbon dioxide (Maicas, 2020; Rai & Jeyaram, 2017). Among yeasts, *S. cerevisiae* is the most widely utilized species in food fermentation due to its rapid fermentation rate, high ethanol tolerance, and production of desirable aroma compounds such as esters and higher alcohols (Parapouli et al., 2020).

In addition to bacteria and yeasts, filamentous fungi have long been employed in food fermentations due to their enzymatic capabilities and their contribution to flavor and texture development (Corbu et al., 2023). Among these, *Penicillium* species such as *Penicillium roqueforti* and *Penicillium camemberti* play a central role in the ripening of blue and soft cheeses, where they generate characteristic flavors, textures, and visual appearance (Łopusiewicz et al., 2020). Similarly, *Aspergillus oryzae* is essential for the production of traditional Asian fermented foods such as soy sauce, miso, and sake, providing amylolytic and proteolytic enzymes that hydrolyze complex carbohydrates and proteins into fermentable sugars and amino acids (Nout & Aidoo, 2011).

These groups collectively illustrate the diversity of microbial contributions to fermented foods. Table 2 provides an overview of the main microbial groups, their fermentation types, associated metabolites, and functional roles.

Table 2 Microbial groups, fermentation types, metabolites, and functional contributions in fermented foods

Microbial Group	**Type of Fermentation**	**Metabolites Produced**	**Contribution to Product Characteristics**	**Example Products**	**References**
LAB *(Lactobacillus, Leuconostoc, Lactococcus, Pediococcus)*	Lactic acid fermentation: Homofermentative (lactic acid) and Heterofermentative (lactic acid, acetic acid, ethanol, CO_2)	Lactic acid, ethanol, CO_2, acetic acid, bacteriocins, vitamins	Acidification and preservation; flavor development; texture modification; enhanced mouthfeel	Yogurt, cheese, sauerkraut, kimchi, sourdough	(Giraffa et al., 2010; Jung et al., 2012; LeBlanc et al., 2015; T. Liu et al., 2020; Wang et al., 2021; Zacharof & Lovitt, 2012)
Yeasts *(Saccharomyces, Brettanomyces, Pichia*)	Alcoholic fermentation	Ethanol, CO_2, higher alcohols, esters, aldehydes	Leavening, carbonation, flavor and aroma complexity	Bread, beer, wine, cider, kombucha, coffee, cocoa	(Che et al., 2024; Gutiérrez-Ríos et al., 2022; Maicas, 2020; S R et al., 2022)
AAB (*Acetobacter, Gluconobacter, Komagataeibacter, Gluconacetobacter*)	Acetic acid fermentation	Acetic acid, gluconic acid, volatile acids, glycerol, exopolysaccharides	Acidity modulation; aroma diversification; product stabilization	Kombucha, vinegar, cocoa, vitamin-enriched foods	(Gomes et al., 2018; Gullo et al., 2018; Shinjoh & Toyama, 2016)
Filamentous Fungi / Molds (*Penicillium, Aspergillus, Rhizopus*)	Organic acid fermentation (citric, fumaric, oxalic, lactic in some species); proteolytic and lipolytic metabolism	Amino acids, fatty acids, esters, pigments, bioactive compounds	Development of flavor, aroma, texture and color	Cheese (blue, Camembert), tempeh, soy sauce, citric acid	(Copetti, 2019; Dufossé et al., 2014; Martín & Coton, 2017; Strong et al., 2022)

Sorghum: Composition, Nutritional Value, and Fermentation Potential

Sorghum (*Sorghum bicolor* L. Moench) is a gluten-free cereal recognized for its strong agronomic performance and adaptability to harsh environments. Sorghum ranks as the fifth most produced grain worldwide after wheat, rice, maize, and barley, with an estimated 59.78 million metric tons produced between 2015 and 2024 (Hamaker et al., 2025; U.S. Department of Agriculture, Foreign Agricultural Service, 2025). Sorghum's ability to thrive under drought and semi-arid conditions has positioned it as a strategic crop of growing importance for global food security and health-oriented product development (Meena et al., 2022).

From a compositional perspective, sorghum is comparable to the other major cereals such as corn. Sorghum provides 8–18% protein, 70–80% carbohydrates, 2–3% lipids, and 6-7% of dietary fiber, along with vitamins, minerals, and phytochemicals (Tanwar et al., 2023). The starch fraction is more resistant to enzymatic digestion than that of other cereals, undergoing limited hydrolysis in the small intestine and reaching the colon, where it is metabolized by gut microbiota to produce short-chain fatty acids with well-documented health benefits (Bojarczuk et al., 2022; Khoddami et al., 2023; Zahid et al., 2025). Sorghum proteins are concentrated in the endosperm and include kafirins (prolamins), glutelins, albumins, and globulins. Kafirins are characterized by hydrophobicity and disulfide cross-linking, which reduce digestibility but also confer unique functional properties such as film-forming ability and thermal stability (Balandrán-Quintana et al., 2019; Pontieri & Del Giudice, 2016). Lipids are primarily concentrated in the germ and show a fatty acid composition comparable to maize oil, dominated by linoleic and oleic acids, with smaller amounts of palmitic and stearic acids (Pontieri & Del Giudice, 2016; Ratnavathi, 2019).

In addition to macronutrients, sorghum is rich in micronutrients and bioactive compounds. It contains B-complex vitamins essential for metabolic processes and minerals such as phosphorus, magnesium, iron, and copper that contribute to bone health, red blood cell production, and enzymatic activity (Tanwar et al., 2023). Sorghum also provides a range of bioactives, including polyphenols, flavonoids, carotenoids, and tocochromanols (α-, β-, γ-, and δ-tocopherol). These compounds have been associated with antioxidant, anti-inflammatory, and other regulatory functions that contribute to the grain's nutritional and functional value (De Morais Cardoso et al., 2017; J. Xu et al., 2021).

Despite its nutritional profile, sorghum use in human foods and animal feeds is limited by anti-nutritional factors such as tannins, phytic acid, trypsin inhibitors, and protein cross-linkers, which reduce protein and mineral bioavailability and decrease feed efficiency (Rashwan et al., 2025). To address the challenges presented by said anti-nutritional factors, multiple processing strategies have been evaluated. Physical methods such as decortication primarily reduce tannin levels, reflecting their concentration in the outer grain layers, while phytates, located in the inner endosperm, are less affected (Sruthi et al., 2021). Extrusion has also been studied, with outcomes depending on sorghum variety and processing conditions (Bhattarai et al., 2025; Mkandawire et al., 2015). Biochemical approaches, including germination and malting, reduce both tannins and phytates through endogenous enzymatic activity and have been reported to enhance mineral bioaccessibility and protein solubility (Atanda Usman, 2018).

Among the strategies evaluated, fermentation has emerged as particularly effective. Available studies demonstrate its ability to enhance nutrient digestibility and reduce anti-nutritional factors**.** For instance, Pranoto et al., (2013) reported that both natural and *Lactobacillus*-mediated fermentation of sorghum flour significantly improved in vitro

protein and starch digestibility after 36 h. Similarly, Putri et al., (2021) observed a reduction in tannin content following 48 h of lactic acid fermentation. Beyond effects on nutrient bioaccessibility, fermentation also modifies sorghum's bioactive profile. Polyphenols can be hydrolyzed into smaller, more bioavailable compounds, such as quercetin, kaempferol, gallic acid, and ellagic acid, through the activity of microbial enzymes like tannases and glycosidases, thereby enhancing antioxidant potential (Garcia-Alonso et al., 2023; K & Gokhale, 2025). In addition to compositional changes, in vivo research has demonstrated broader implications of fermentation. Ofosu et al., (2023) reported that feeding mice fermented sorghum increased butyrate production, promoted beneficial shifts in the gut microbiota, and reduced opportunistic pathogens, indicating that fermentation can influence both nutritional outcomes and host–microbe interactions.

Taken together, the evidence suggests that fermentation is not only a strategy to address sorghum's nutritional limitations but also a means of enhancing its functional properties and supporting its application in health-oriented foods.

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Chapter 2 - Research Gap

Sorghum (*Sorghum bicolor* (L.) Moench) is a widely cultivated grain valued for its resilience to heat and drought environments (Meena et al., 2022). In addition to its agronomic advantages, sorghum is naturally gluten-free and contains bioactive compounds such as polyphenols that have been associated with potential health benefits (Stefoska-Needham, 2024). Despite these positive attributes, sorghum remains underutilized for human consumption in Western food systems due to intrinsic grain characteristics such as the presence of antinutritional factors and relatively low protein digestibility. Fermentation is an ancient processing strategy widely used in cereal grains to enhance nutritional quality, improve digestibility, and develop desirable functional and sensory properties (Adebo et al., 2022). Both submerged fermentation systems, commonly used in fermented beverages, and solid-state fermentation (SSF) have demonstrated the potential to improve grain nutritional characteristics through microbial metabolism and biochemical transformations.

Recent studies have explored sorghum as a substrate for fermented beverages, including applications with kombucha cultures, demonstrating the feasibility of producing sorghum-based functional drinks (Kiptanui et al., 2022; Nelson et al., 2023). However, microbial dynamics during sorghum kombucha fermentation and the influence of sorghum variety on fermentation outcomes remain underexplored. Furthermore, studies evaluating whether sorghum kombucha can modulate microbial communities are limited.

Solid-state fermentation has been reported to reduce antinutritional factors and enhance nutrient bioaccessibility (Chen & Zhu, 2015; Terefe et al., 2021). Nevertheless, most studies have focused on sorghum flour, while research on whole sorghum grain fermentation remains

limited. In addition, differences in fermentation responses among sorghum varieties and under varying moisture conditions remain poorly understood.

Together, these gaps highlight the need for studies evaluating fermentation strategies for sorghum across both submerged and solid-state systems while considering the influence of sorghum variety on fermentation dynamics. Addressing these gaps will contribute to expanding sorghum grain applications as fermented foods, functional beverages, and nutritionally improved grain ingredients. Therefore, the objective of this research was to explore fermentation strategies to broaden the use of sorghum grains, using both submerged and solid-state fermentation systems, and evaluate how sorghum variety and fermentation conditions influence microbial activity and nutritional quality.

To achieve this objective, the following specific objectives were addressed:

1. Evaluate different sorghum grain varieties as substrates for kombucha fermentation by characterizing fermentation dynamics, microbial growth, and microbial community composition.
2. Explore the potential effects of sorghum kombucha consumption by evaluating fecal microbial community composition and *Enterobacteriaceae* shedding in an obesity intervention pig model.
3. Assess the impact of lactic acid bacteria solid-state fermentation on whole sorghum grains, focusing on microbial growth, phytic acid reduction, and in vitro protein digestibility under different sorghum varieties and moisture conditions.

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Chapter 3 - Microbial Profiling of Sorghum-based Kombucha

Abstract

Kombucha is a fermented beverage traditionally prepared with black or green tea, sugar, and a Symbiotic Culture of Bacteria and Yeast (SCOBY). It has gained attention for its functional properties, including antioxidant, antimicrobial, and probiotic potential. Alternative substrates have been tested to diversify the composition of kombucha and enhance beneficial properties; Sorghum (*Sorghum bicolor (L.)* Moench*),* a gluten-free cereal rich in polyphenols, is a promising candidate for "functional fermentations". Hence, this study aimed to use and characterize three sorghum varieties as alternative fermentation substrates for kombucha production. Over 7 days, pH, soluble solids (°Brix), and microbial counts were recorded. Shotgun metagenomic sequencing was conducted at the end of the fermentation to characterize the abundance and taxonomic profile of the microbial communities. All treatments exhibited significant decreases ($p < 0.05$) in pH and °Brix during fermentation. By day 7, the overall microbial populations increased significantly across all treatments ($p < 0.05$), with the control showing the highest counts. Metagenomic analysis identified *Brettanomyces* as the dominant genus in the starter pellicle, which remained predominant throughout the fermentation process. Our study offers an initial framework on the utilization of sorghum as an alternative substrate for food and beverage fermentations.

Introduction

In recent years, there has been a growing interest in functional foods and beverages. As consumers become increasingly health-conscious, they actively seek products that not only meet dietary needs but also contribute to overall well-being. Kombucha, a fermented tea of Asian origin (Yu-cheng Sui et al., 2018), has gained popularity in the West due to its anti-inflammatory and antioxidant properties (Villarreal-Soto et al., 2018; Watawana et al., 2015) and probiotic traits (Jayabalan et al., 2014; J. Xu et al., 2021). The traditional drink is produced from green and/or black tea that has been fermented with a Symbiotic Culture of Bacteria and Yeast (SCOBY). This cellulose-based starter contains unique combinations of bacteria and yeast, most commonly acetic acid bacteria (AAB), such as *Komagataeibacter*, *Gluconobacter*, and *Acetobacter* species (De Roos & De Vuyst, 2018), lactic acid bacteria (LAB), including *Lactobacillus* spp., *Lactococcus* spp., *Leuconostoc* spp. (Chakravorty et al., 2016) and yeasts such as *Brettanomyces spp., Saccharomyces spp., and Schizosaccharomyces spp.* (Antolak et al., 2021; Coton et al., 2017).

Recent studies have explored alternative substrates for kombucha fermentation to diversify sensory and functional properties. (Khosravi et al., 2019) evaluated date syrup and compared it to traditional kombucha, observing similar physicochemical characteristics. (Rocha-Guzmán et al., 2023) used maqui juice for kombucha fermentation and reported an accumulation and enhancement of phenolic compounds stability. Similarly, another study (Kushargina et al., 2024) assessed telang flower tea as an alternative substrate and reported increased antioxidant properties.(C. Xu et al., 2024*)* also observed increased flavonoid and polyphenol content when evaluating *Flos sophorae* and elm fruits kombucha compared to traditional kombucha. These

findings highlight the potential for utilizing various non-traditional sources in kombucha production, often aiming to leverage unique nutritional or functional properties.

Sorghum (*Sorghum bicolor* (L.) Moench), the world's fifth most important cereal crop, is widely cultivated for both food and feed applications, and the United States is the largest producer (Hossain et al., 2022a; U.S. Department of Agriculture, Foreign Agricultural Service, 2025). This gluten-free versatile grain is recognized for its ability to thrive under adverse growing conditions (Hossain et al., 2022b), its diverse phytochemical profile, including phenolic acids, flavonoids, and its high dietary fiber content. These attributes make sorghum a promising candidate for the development of functional foods and fermentation (De Morais Cardoso et al., 2017; Sang et al., 2008; Yan et al., 2011).

Therefore, in this study, three different sorghum varieties were evaluated as alternative substrates for kombucha fermentation. Parameters such as microbial dynamics, pH, total soluble solids (°Brix), as well as community abundance and taxonomic profiles, were reported and compared to those of traditional tea-based kombucha.

Materials and Methods

Sorghum Varieties and SCOBY

Three whole-grain sorghum varieties were used in this study: white sorghum (NLSC-WSWG-0010), a non-tannin type; sumac sorghum (NLSC-SUSWG-0018), high in tannins and polyphenols (Kaufman et al., 2013); and waxy sorghum (NLSC-WBSWG-0014L), an amylopectin-rich starch variety *(37)*. All sorghum samples were sourced from Nu Life Market (Scott City, KS, USA). A commercial organic kombucha SCOBY (Fermentaholics, Clearwater, FL, USA), consisting of cellulose pellicle and liquid phase, was used as the starter culture.

Kombucha Preparation

For sorghum kombucha: whole sorghum grains (230 g) were rinsed three times with deionized (DI) water until the wash water was clear. The grains were soaked in 690 ml of DI water at room temperature (22 ± 2 °C) for 16 h and covered with a coffee filter. The following day, 480 ml of the soaked extract was strained, brought to a rolling boil, and held for 5 min to minimize microbial contamination. While still hot, granulated cane sugar (217 g) was added and stirred until fully dissolved. Subsequently, 1892 ml of DI water was added. When the extract temperature reached <35 °C, the SCOBY (pellicle and liquid phase) was introduced to the fermentation jar, covered with a coffee filter, and incubated at 27 °C for 7 days in the dark.

For comparison, a traditional tea-based kombucha was prepared according to a recipe provided by a commercial collaborator. Black tea (8 g) and green tea (17 g) were steeped in 473 ml of hot deionized water for 5 min and then removed. The infusion was transferred to a 1-gallon glass jar, mixed with 108 g of cane sugar and 1420 ml of deionized water, and stirred until the sugar was dissolved. After cooling to <35 °C, the SCOBY was added to the jar, covered with a coffee filter, and incubated at 27 °C in the dark for 7 days in the dark. All treatments and controls were performed in triplicate.

Determination of pH and Soluble Solids

An aliquot (5 ml) of fermented liquid sample was collected from each jar at four points in time (days 0, 3, 5, and 7). The pH of each sample was measured using a benchtop pH/mV meter (SevenExcellence S400, Mettler Toledo, Columbus, OH, USA). Total soluble solids (°Brix) were determined with a handheld refractometer (°Brix Refractometer, Extech, Nashua, NH, USA).

Microbiological Analysis

The same sampling days were used for microbial analysis, where 1 ml of sample was aseptically collected from each fermentation jar, serially diluted in 0.1% peptone water (Bacto, Sparks, MD, USA), and plated in duplicate. Acetic acid bacteria (AAB) were enumerated on Wallerstein Laboratory Differential Agar (WL; HiMedia, Mumbai, India) and incubated at 27 °C for 48 h. Lactic acid bacteria (LAB) were enumerated on de Man, Rogosa, and Sharpe (MRS) agar (Difco, Detroit, MI, USA), and incubated at 35 °C for 72 h. Yeasts and molds (YM) were enumerated on Petrifilm™ Yeast and Mold Count Plates (Neogen, Saint Paul, MN, USA) and incubated at 27 °C for up to 5 days.

DNA Extraction, Shotgun Sequencing, and Bioinformatics Analysis

Genomic DNA was extracted from the commercial starter culture used for fermentation (pellicle and liquid, day 0) and the day-7 liquid phase of the sorghum kombucha. Microbial composition analyses were performed only on the sorghum kombucha liquid, as this fraction represents the final product consumed. The tea control was not sequenced, as the analysis focused on sorghum-specific shifts relative to the starter. For the starter culture, 5 g of the central region of the SCOBY pellicle and 10 ml of liquid were collected. At the end of fermentation (day 7), only liquid samples from the sorghum kombucha were collected, and the resulting liquid was centrifuged. The resulting pellet was then used for DNA extraction.

DNA was extracted using the PowerSoil Pro Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions, and the concentrations were determined using a Qubit 4 Fluorometer (Q32857, Invitrogen, MA, USA). The extracts were stored at −20 °C until further use. Libraries were prepared with the Nextera DNA Flex Library Preparation Kit (Illumina, San Diego, CA, USA), quantified, pooled at equimolar concentrations, and sequenced on a NovaSeq

6000 platform (Illumina) using 2 × 150 bp paired-end chemistry (Novogene, Sacramento, CA, USA). Raw reads were quality-filtered with readfq (v10.5; https://github.com/cjfields/readfq) to remove low-quality bases, ambiguous reads, and adapter contamination. Host-related sequences were filtered by alignment with Bowtie2 (v2.4.5; http://bowtie-bio.sourceforge.net/bowtie2) using the parameters --end-to-end, --sensitive, -I 200, and -X 400. Clean reads were assembled with MEGAHIT (v1.2.9) using the meta-large preset. Open reading frames (ORFs) were predicted with MetaGeneMark (v3.38; ≥500 bp; sequences <100 nt excluded). Taxonomic annotation was performed in MEGAN (v6.24.2) using the lowest common ancestor (LCA) algorithm based on DIAMOND (v2.0.15; blastp, e ≤ 1e−5) alignments against the NCBI NR database. Relative species abundances were calculated for downstream analysis. Group-level differences were assessed with Metastats and LEfSe (LDA score ≥ 4). Principal Coordinates Analysis (PCoA) based on Bray–Curtis dissimilarities was performed to visualize differences in microbial community composition among treatments.

Experimental Desing and Statistical Analysis

Four treatment combinations were evaluated in a completely randomized design (CRD) with a one-way treatment structure consisting of three sorghum varieties and one traditional tea based control. Each treatment was conducted in triplicate, resulting in a total of 12 experimental units. Response variables (microbial counts, pH, and °Brix) were measured at 0, 3, 5 and 7 days.

Data were analyzed using linear mixed-effects models to account for repeated measures over time. Fixed effects included treatment, sampling time, and their interaction (treatment x day) with replicates included as random effect. Estimated marginal means were obtained and pairwise comparison were performed within each sampling day using Sidak-adjusted tests. All statistical analyses were performed in R (version 4.5) using the lme4 package for fitting linear

mixed models (Bates et al., 2015), and the emmeans package for estimated marginal means and comparisons (Lenth & Piaskowski, 2017). Statistical significance was set at $\alpha = 0.05$ for all tests.

Results

Changes in pH and Soluble Solids

Table 3 summarizes the physicochemical changes observed during the fermentation of kombucha. All treatments showed a significant decrease in pH over time ($p < 0.05$). By day 7, sorghum kombucha samples had significantly lower pH values than the control ($p < 0.05$): 3.31 ± 0.02 (control), 2.96 ± 0.13 (white), 3.07 ± 0.09 (waxy), and 3.04 ± 0.01 (sumac), all within the range considered safe for consumption (2.5–4.2) (De Miranda et al., 2022). Soluble solids (°Brix) also declined significantly across treatments ($p < 0.05$). By day 7, the control reached a lower °Brix value (5.70 ± 0.55) than the sorghum treatments: 7.66 ± 0.28 (white), 7.57 ± 0.35 (sumac), and 7.53 ± 0.05 (waxy).

Microbial Dynamics

Microbial population dynamics during fermentation are summarized in Table 4. At day 0, initial microbial counts across treatments were 3.22 ± 0.37 log CFU/ml for AAB, 3.28 ± 0.63 for LAB, and 3.10 ± 0.50 for YM. All three microbial groups increased significantly ($p < 0.05$). in both the control and sorghum kombucha samples over time.

On days 3 and 5, control samples exhibited higher AAB ($p < 0.05$) counts than all sorghum samples, while by day 7, AAB counts in the control and waxy samples were similar and significantly higher ($p < 0.05$) than those in white and sumac samples. LAB counts increased significantly from day 0 to day 7 across all treatments ($p < 0.05$). By the end of fermentation, control and waxy kombucha samples showed higher counts ($p < 0.05$) than white and sumac

kombucha samples. The control reached the highest counts ($p < 0.05$) for YM by day 7, while among sorghum samples, waxy and sumac exhibited higher YM levels than white samples ($p < 0.05$).

Microbial Diversity and Composition

In the starter pellicle (day 0), *Brettanomyces* was the dominant genus (> 75%), followed by *Liquorilactobacillus* (<10%), *Komagataeibacter* (<5%), and *Pediococcus* (<5%) (Figure 1A). In the starter liquid, *Komagataeibacter* (~30%) was the most abundant genus, followed by *Acetobacter* (~10%) and *Gluconacetobacter* (<10%). All other low-abundance taxa were grouped as "Others". Across all sorghum kombucha samples, *Brettanomyces* was the dominant genus also at day 7. White and sumac exhibited similar profiles, with *Brettanomyces* dominant (>75%) and a smaller proportion of *Zygosaccharomyces*, *Komagataeibacter*, and *Pediococcus* (<5%), while waxy kombucha sample had *Brettanomyces* as the most abundant genus (~60%), but higher relative abundances of AAB (*Komagataeibacte* ~15%, *Acetobacter* ~10%, *Gluconacetobacte* <5%) and acid-tolerant yeast, (*Pichia* <10%) as compared to white and sumac samples.

When analyzing beta diversity using Principal Coordinates Analysis (PCoA) based on Bray-Curtis dissimilarity metrics (Figure 1B), the starter culture showed clear separation between phases. At day 0, the liquid fraction clustered apart from the pellicle, confirming the diverse relative abundance profiles observed (Figure 1A). At day 7, white and sumac sorghum kombucha samples clustered closely, indicating similar community structures, whereas waxy samples grouped apart, consistent with the distinct taxonomic composition previously observed.

Discussion

The present study evaluates the performance of three sorghum varieties for kombucha fermentation, establishing an initial framework for using sorghum grains as alternatives substrates for kombucha fermentation. While several studies have reported the use of alternative substrates for kombucha fermentation, the specific effects of different sorghum varieties on microbial growth dynamics, pH and °Brix, as well as microbial composition shift relative to the initial starter culture remain unexplored. Similar to other fermentation systems, pH and °Brix have been used as indicators of fermentation progress and microbial activity (Chong et al., 2024; Svendsen et al., 2015; Tejedor-Calvo & Morales, 2023). Consistent with our results, a decline in pH during fermentation has been frequently reported and is largely attributed to the accumulation of organic acids produced by the kombucha microbial consortium (Chong et al., 2024; Villarreal-Soto et al., 2018). Nevertheless, differences in pH between fermented samples are usually not determined solely by microbial activity, but also by the intrinsic buffering capacity of the substrate (Torija et al., 2003). This interaction has been observed by Khosravi et al., (2019). The authors noticed that both microbial metabolism and buffering properties of date syrup influenced changes in pH and titratable acidity compared with traditional tea kombucha. Similarly, De Lima et al., (2025) reported substrate-dependent acidification patterns when fermenting passion fruit leaves. In our study, we also observed a decrease in °Brix across all tested samples. Previous research has reported similar decreases and linked these phenomena to yeast-mediated sucrose hydrolysis. Do et al., (2024) reported a similar reduction in °Brix in papaya-based kombucha after 14 days of fermentation, while Ayed et al., (2017) observed a decrease in °Brix in grape juice kombucha.

To our knowledge, the present research is one of the few studies evaluating sorghum as a substrate for kombucha fermentation. The microbial counts observed in our study were consistent with those reported in previous kombucha research. Cao et al., (2025) fermented blackberry-based kombucha for 7 days at 28 °C and 37 °C and reported yeast levels of 6.5–7 log CFU/ml and bacterial counts of 5.5–6 log CFU/ml, values comparable to our findings. Similarly, Velicanski et al., (2013) documented comparable counts in kombucha fermented with medicinal herbs, such as lemon balm, thyme, and peppermint, at 28 °C for 7 days. Our results suggest that sorghum extract can provide an environment capable of supporting typical kombucha microbial populations.

The dominance of *Brettanomyces* in both the starter and sorghum kombucha samples aligns with previous reports from traditional black and green tea kombucha (Reva et al., 2015), large-scale experiments (Coton et al., 2017), and even alternative kombucha substrates, such as telang flower tea (Kushargina et al., 2024). Likewise, the presence of *Komagataeibacter* and related AAB genera is consistent with previous research, which highlights their well-established roles in acetic acid production and cellulose synthesis, driving SCOBY formation (Villarreal-Soto et al., 2020). Likewise, Landis et al., (2022) identified *Brettanomyces* and *Komagataeibacter* as the most frequent genera across commercial kombucha products in the United States, with the relative abundance of *Komagataeibacter* ranging widely from 1% to 52%. (Sharifudin et al., 2021*)* reported comparable community patterns when fermenting papaya-based substrates with commercial kombucha starters.

Liquorilactobacillus (<5%) and *Pediococcus* (<5%) were the only LAB genus detected in our study. Both genera are commonly associated with plant fermentations and silage (Fugaban et al., 2022; Liu et al., 2023), and although they have been reported in kombucha (Diguță et al.,

2020; Larini et al., 2024), they have been less frequently reporteed than other LAB, such as *Lactobacillus* and *Oenococcus* (Sogin & Worobo, 2024; Tran et al., 2020).

Conclusions

This study establishes an initial framework for utilizing sorghum grains as alternative substrates for kombucha fermentation. Overall, variability is expected among kombucha studies, mostly coming from differences in sampling time, substrate composition (sorghum vs. tea), inoculum characteristics, and production conditions can all influence the abundance of microbial groups. In our study, we observed that regardless of the grain variety, sorghum kombucha fermentation exhibited microbial profiles comparable to those of traditional kombucha, indicating that sorghum can support a similar beneficial microbial community. These findings highlight the potential of sorghum to enhance the functionality and nutritional value of fermented beverages without significantly altering product characteristics and microbial composition.

Acknowledgments

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Tables and Figures

Table 3 Physicochemical changes during sorghum and control kombucha fermentation.

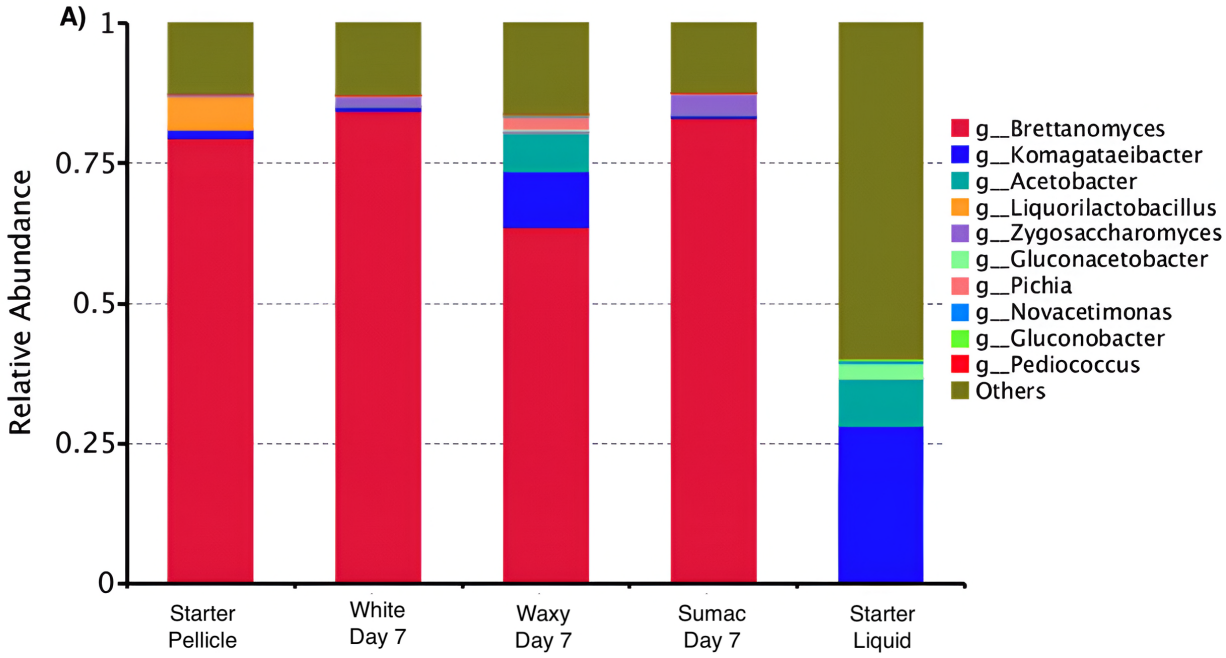
Parameter	Day	Control	White	Waxy	Sumac
pH	0	3.54 ± 0.07^{aB}	3.21 ± 0.05^{bA}	3.51 ± 0.04^{aA}	3.42 ± 0.11^{aA}
	3	3.74 ± 0.04^{aA}	3.15 ± 0.05^{cA}	3.31 ± 0.04^{bB}	3.27 ± 0.07^{bB}
	5	3.39 ± 0.03^{aC}	3.02 ± 0.09^{cB}	3.16 ± 0.07^{bC}	3.13 ± 0.02^{bC}
	7	3.31 ± 0.02^{aC}	2.96 ± 0.13^{bB}	3.07 ± 0.09^{bD}	3.04 ± 0.01^{bC}
°Brix	0	7.27 ± 0.20^{bA}	8.00 ± 0.00^{aA}	8.03 ± 0.05^{aA}	8.03 ± 0.05^{aA}
	3	6.97 ± 0.05^{bB}	7.93 ± 0.05^{aA}	8.00 ± 0.05^{aA}	7.93 ± 0.10^{aA}
	5	6.63 ± 0.37^{bB}	7.77 ± 0.05^{aB}	7.83 ± 0.11^{aB}	7.77 ± 0.05^{aB}
	7	5.70 ± 0.55^{bB}	7.66 ± 0.28^{aB}	7.57 ± 0.35^{aC}	7.53 ± 0.05^{aC}

Different lowercase letters (a–c) within a row indicate significant differences among treatments within the same day; different uppercase letters (A–D) indicate significant differences among days within the same treatment ($\alpha = 0.05$). Values are means ± SD ($n = 3$).

Table 4 Microbial counts (log CFU/ml) of acetic acid bacteria (AAB), lactic acid bacteria (LAB), and yeasts and molds (YM) during sorghum and control kombucha fermentation.

Microorganism	Day	Control	White	Waxy	Sumac
AAB	3	6.96 ± 0.32^{aB}	3.93 ± 0.70^{bB}	4.70 ± 0.35^{bB}	4.80 ± 0.36^{bB}
	5	7.51 ± 0.12^{aA}	5.90 ± 0.30^{bA}	4.87 ± 0.61^{cB}	6.50 ± 0.44^{bA}
	7	7.61 ± 0.04^{aA}	6.10 ± 0.52^{bA}	6.87 ± 0.06^{abA}	6.27 ± 0.85^{bA}
LAB	3	6.76 ± 0.08^{aA}	6.23 ± 0.06^{abA}	6.67 ± 0.46^{aA}	5.50 ± 1.04^{bB}
	5	7.43 ± 0.12^{aA}	6.48 ± 0.38^{aA}	6.93 ± 0.31^{aA}	6.27 ± 0.38^{aA}
	7	7.63 ± 0.07^{aA}	6.18 ± 0.50^{bA}	6.87 ± 0.21^{abA}	6.37 ± 0.38^{bA}
YM	3	6.54 ± 0.23^{aB}	5.72 ± 0.33^{aA}	4.33 ± 0.80^{bC}	4.33 ± 0.46^{bC}
	5	7.64 ± 0.20^{aB}	5.90 ± 0.20^{bA}	6.03 ± 0.59^{bB}	5.97 ± 0.61^{bB}
	7	7.92 ± 0.25^{aA}	5.60 ± 0.17^{cA}	6.87 ± 0.15^{bA}	6.60 ± 0.17^{bA}

Different lowercase letters (a–c) indicate significant differences among treatments within the same day; different uppercase letters (A–C) indicate significant differences among days within the same treatment ($\alpha = 0.05$). Values represent means ± standard deviation ($n = 3$). Initial microbial counts were: AAB, 3.22 ± 0.37; LAB, 3.28 ± 0.63; YM, 3.10 ± 0.50.


A)
Relative Abundance
1
0.75
0.5
0.25
0
Starter Pellicle
White Day 7
Waxy Day 7
Sumac Day 7
Starter Liquid
g__Brettanomyces
g__Komagataeibacter
g__Acetobacter
g__Liquorilactobacillus
g__Zygosaccharomyces
g__Gluconacetobacter
g__Pichia
g__Novacetimonas
g__Gluconobacter
g__Pediococcus
Others

987

988

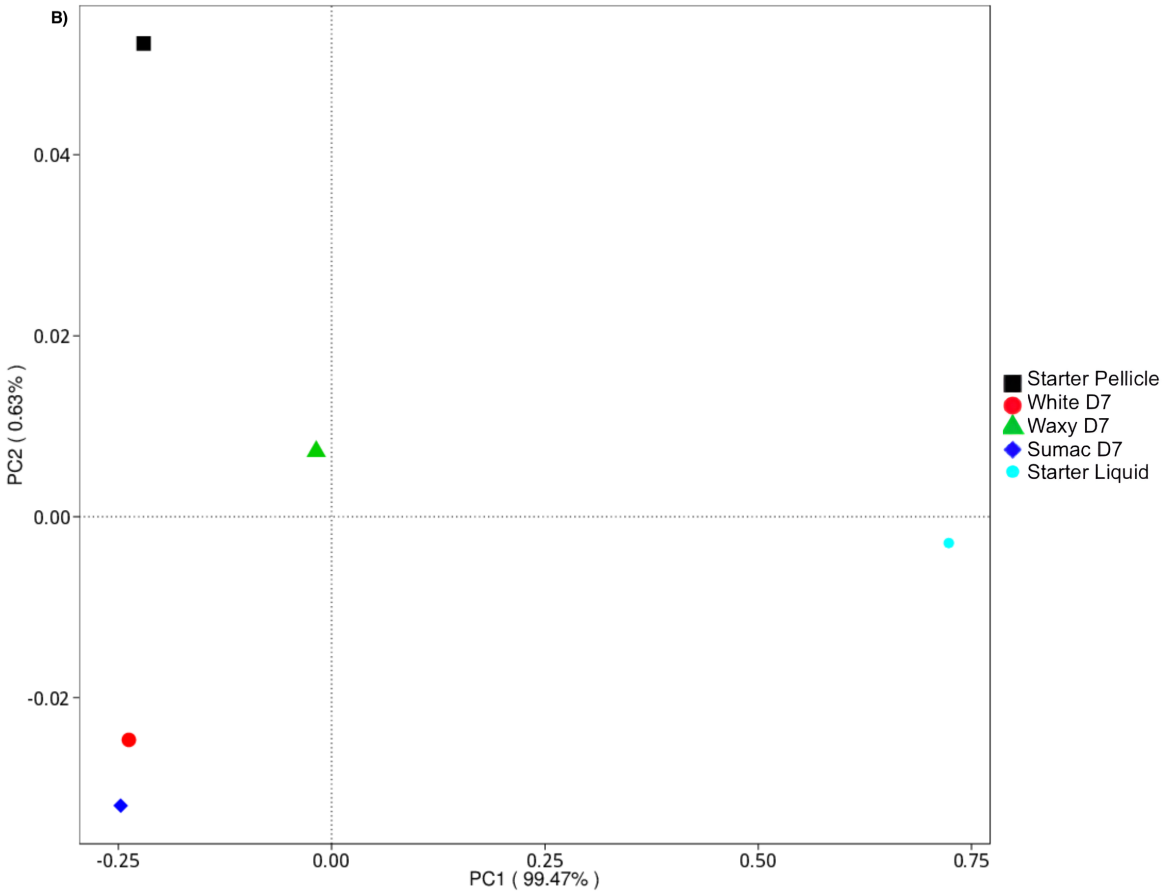


Figure 1 Microbial Diversity and Composition. (A) Relative abundance of microbial genera in the commercial starter culture (starter liquid and SCOBY pellicle, day 0) and sorghum kombucha samples at day 7. Only the ten most abundant genera are shown; all others are grouped as "Others." (B) Principal Coordinates Analysis (PCoA) of microbial communities based on Bray–Curtis distance. Axes indicate the percentage of variation explained by each principal coordinate. Samples clustering together share similar microbial composition, whereas separated samples indicate distinct communities.

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Chapter 4 - Effect of Sorghum Kombucha Supplementation on *Enterobacteriaceae* Shedding and Fecal Microbiota in Ossabaw pigs During Early Weight Management

Abstract

Fermented foods such as kombucha are gaining attention as healthy dietary strategies. Sorghum is a climate-resilient grain with promising characteristics for functional foods and fermentation. This study evaluated the effects of sorghum kombucha intervention on fecal *Enterobacteriaceae* shedding and microbiota in an obesity intervention model using Ossabaw pigs. Sixteen pigs (8 male, 8 female) were fed diets (standard and western-style diet) to induce lean or obese body types. At week 17, the animals start receiving sorghum kombucha or isocaloric tea (500 mL twice daily). Fecal samples were collected at weeks 17, 21, and 23 and analyzed for *Enterobacteriaceae* counts and metagenomics. Kombucha supplementation significantly reduced *Enterobacteriaceae* shedding ($p < 0.05$) in both lean and obese pigs as compared to isocaloric tea group. Shotgun metagenomic profiling identified *Treponema, Streptococcus,* and *Prevotella* as dominant genera across treatments and sampling weeks. Beta diversity analysis showed that host body type (lean vs. obese) had a stronger influence on microbial community composition than tea treatment (kombucha vs. isocaloric tea). Our study offers an initial framework to understand the potential health benefits of sorghum kombucha consumption.

Introduction

The consumption of fermented foods and beverages has been associated with changes in microbiota composition and metabolic activity (Shah et al., 2023; Valentino et al., 2024).

The effects of fermented foods are not solely dependent on viable microorganisms but also on fermentation-derived metabolites, including organic acids, modified polyphenols, and microbial metabolites, which can shape microbial communities (Marco et al., 2017; Stiemsma et al., 2020; Yang et al., 2023). These diverse communities can support balanced metabolite production and limit the accumulation of potentially harmful compounds (Chang, 2024; Shtossel et al., 2024).

Kombucha is a beverage traditionally produced by fermenting sweetened tea with a symbiotic culture of bacteria and yeast (SCOBY) (Villarreal-Soto et al., 2018). Kombucha contains organic acids, antioxidants, and microbial-derived metabolites that have been reported to exhibit antimicrobial and anti-inflammatory properties (Ecklu-Mensah et al., 2024; Onsun et al., 2025). Consequently, kombucha has emerged as a fermented beverage of interest for its potential to modulate microbiota health (Costa et al., 2022; Li et al., 2025). While kombucha is traditionally made from black or green tea, alternative substrates have been evaluated to diversify sensory and functional properties, with sorghum being a viable substrate for kombucha fermentation (Kiptanui et al., 2022; Nelson et al., 2023).

Sorghum (*Sorghum bicolor* (L.) Moench) is a gluten-free grain characterized by the presence of phenolic acids and flavonoids that contribute to its antioxidant capacity (De Morais Cardoso et al., 2017). In addition to these bioactive compounds, sorghum contains dietary fiber and complex carbohydrates that can serve as substrates for microbial fermentation (Garzón et al., 2024).

In order to evaluate the effects of dietary intervention on microbiota health, Ossabaw pigs have been frequently used as translational model (Matthan et al., 2018; Zhang et al., 2021). These pigs share key anatomical and microbial similarities with humans, making them suitable for investigating host body type dependent responses to nutritional interventions (Zhang et al.,

2021). Therefore, the goal of this study was to evaluate *Enterobacteriaceae* shedding and characterize fecal microbiota of lean and obese Ossabaw pigs during a 6-week intervention diet with sorghum kombucha or isocaloric tea.

Material and Methods

Study Duration and Locations

This study was conducted from February to July 2024 over a period of 23 weeks. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC protocol #4831-LARC) at Kansas State University (Manhattan, KS). Ossabaw pigs were initially housed at CorVus (Crawfordsville, IN; AAALAC-accredited) and at week 12 of the study they were transferred to the Large Animal Research Center (LARC; AAALAC-accredited) at Kansas State University for the remainder of the study.

Animals and Dietary Allocation

Sixteen Ossabaw pigs (8 males and 8 females; mean ± SD body weight = 8.83 ± 1.98 kg) of around 14 weeks of age were selected for the study and housed in sex pairs. Within each sex, pigs in the first and fourth weight quartiles were matched, whereas those in the second and third quartiles were randomly assigned to pens. Animals were divided into two dietary groups. Eight received a standard chow diet (LabDiet Mini-Pig HF Grower 5L80, Purina Test Diet, Richmond, IN) formulated to support healthy body weight (16 % protein, 72 % carbohydrate, 3 % fat, up to 1,650 kcal/day) and grouped as “lean”. The other 8 were fed a hypercaloric Western-style diet formulated by CorVus (MetS; KT324; Corvus Biomedical, IN) containing 8 % protein, 46 % carbohydrate (20 % from fructose), 46 % fat (56 % trans fats), and 2 % cholesterol (up to 3,400 kcal/day), and grouped as “obese”. Diets were randomly assigned to ensure sex balance across groups (n = 8 per diet; sex balanced). After 12 weeks, the pigs were transferred to the LARC

facility. The same diet regimen (standard and western diet) continued for an additional five weeks. Feed was offered twice daily (08:00 and 16:00 h) in a four-bowl feeder with a central barrier. After acclimation, feed was presented in two diagonally opposed bowls. Water was available ad libitum, and animals were maintained under a light–dark cycle.

Treatments: Kombucha and Isocaloric tea

After 16 weeks, obese pigs were gradually transitioned to the same diet as lean pigs, using a staged protocol: days 1–3 (50:50 chow: Western), days 4–6 (75:25), and days 7–9 (100 % standard chow). By week 17, all pigs consumed the same diet. Subsequently, pigs underwent a 6-week intervention (week 18-23). Within each group (lean and obese), animals were randomized into two treatments (n = 8 per treatment, sex-balanced): kombucha or isocaloric tea was offered twice daily (500 mL after each meal). Both beverages were previously developed and characterized (Nelson et al,. 2023) and the isocaloric tea was matched for energy content (0.03 kcal/mL). A summary timeline of the experiment is given in Figure 2.

Microbiological Analysis

During weeks 17, 21, and 23 of the study, feces were collected from each pig (n = 16 per time point; 48 total samples) and transferred into filtered stomacher bags. Samples (10 g) were homogenized in 90 mL of Tryptic Soy Broth (TSB; BD, Franklin Lakes, NJ, USA) with phosphate buffer (TSB+PO_4), consisting of 30 g TSB/L, 2.31 g KH_2PO_4/L, and 12.54 g K_2HPO_4/L, using the protocol adapted from Harrison et al. (2023). Serial dilutions were prepared, 1 mL aliquots from each dilution were plated onto *Enterobacteriaceae* Petrifilm™ (Neogen®, Lansing, MI, USA) and incubated at 37°C for 24 hours. After incubation, colonies were enumerated according to the manufacturer's interpretation criteria. Red colonies with or

without yellow zones and/or gas bubbles were considered *Enterobacteriaceae.* Counts were expressed as log CFU/g of feces.

DNA Extraction and Shotgun Metagenomic Sequencing

Following microbial enumeration, fecal samples were pooled (10 g total, 5 g from each subsample) and homogenized based on treatment (kombucha or isocaloric tea), body type (lean or obese), sex (male or female), and sampling week (17, 21, 23), yielding a total of 24 samples. From each composite sample, 250 mg was used for DNA extraction using the DNeasy PowerSoil Pro Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. DNA concentrations were quantified using a Qubit 4 fluorometer (Invitrogen, MA, USA), and samples were stored at -20°C until further processing. DNA libraries were prepared with the Nextera DNA Flex Library Preparation Kit (Illumina, USA), quantified individually, and assessed for quality. Libraries were pooled at equimolar concentrations. Metagenomic analysis was performed on a NovaSeq 6000 platform (Illumina, USA) using 2×150 bp paired-end reads

Metagenomic Assembly and Taxonomic Annotation

Bioinformatics analysis involved preprocessing raw Illumina sequencing data using Fastp (https://github.com/OpenGene/fastp) to obtain clean data, and Bowtie2 software (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml) to filter host contaminations. MEGAHIT software was used for assembly analysis of clean data. Contigs (scaftigs ≥ 500 bp) were then subjected to open reading frame (ORF) prediction using MetaGeneMark (http://topaz.gatech.edu/GeneMark/). Predicted ORFs were clustered using CD-HIT (http://www.bioinformatics.org/cd-hit/) to remove redundancy, resulting in a non-redundant gene catalog (unigenes). Unigenes were aligned against the NCBI NR protein database (https://www.ncbi.nlm.nih.gov/) using DIAMOND (https://github.com/bbuchfink/diamond) with the following parameters: blastp, e-value < 1e-5

(Buchfink et al., 2015). Taxonomic assignments were inferred based on best-hit matches and associated taxonomic identifiers, retaining hits within a 10-fold range of the best e-value. Genus-level relative abundances were calculated for each composite sample.

Within-sample (α-) diversity was assessed using Shannon indices, and between-sample (β) diversity was evaluated via Bray–Curtis dissimilarities and visualized by principal coordinate analysis (PCoA). Due to the nature of the samples analyzed (composite samples), diversity was interpreted descriptively. All visualizations were conducted in R (version 4.5) using vegan and rstatix packages.

Experimental Desing and Statistical Analysis

Microbial counts (log CFU/g) were evaluated using a complete randomized design (CRD) with a 2 x 2 x 3 factorial structure consisting of two treatments (kombucha tea and isocaloric tea), two body types (lean and obese) and three sampling points (weeks 17, 21 and 23). Fixed effects included treatment, body type, sampling point their interactions, and sex as a covariate. Each pig (n = 16) was sampled across the three sampling points, resulting in a total of 48 observations. Data was analyzed using a linear mixed-effects model in R (version 4.5) with the glmmTMB package (Brooks et al., 2017). Pig ID was modeled as random effects to account for repeated measures (i.e., with a compound symmetry covariance function). Type III ANOVA was used to test fixed effects and interactions; post hoc pairwise comparisons of estimated marginal means were performed using the the emmeans package with Sidak-adjusted p-values (Lenth & Piaskowski, 2017). Statistical significance was set at $\alpha = 0.05$ for all tests.

Results and Discussion

Enterobacteriaceae Microbial Analysis

The effects of the 6-week kombucha or isocaloric tea interventions on *Enterobacteriaceae* population in lean and obese Ossabaw pigs are shown in Table 5. Lean pigs fed the isocaloric tea group had significantly higher ($p < 0.05$) *Enterobacteriaceae* counts at week 17 (6.60 ± 0.55 log CFU/g) and week 21 (7.07 ± 0.59 log CFU/g), compared to lean pigs fed kombucha (5.78 ± 0.18 log CFU/g and 6.28 ± 0.23 log CFU/g, respectively). No differences were observed between treatments at week 23. Conversely, in obese pigs, *Enterobacteriaceae* levels were similar between groups at week 17 and 21. By week 23 counts were significantly higher ($p < 0.05$) for the isocaloric tea group (6.97 ± 0.15 log CFU/g), as compared to the kombucha group (6.39 ± 0.42 log CFU/g). These observations highlighted a positive effect of sorghum kombucha in decreasing *Enterobacteriaceae* counts based on pig body type (lean vs obese).

Our observed reduction in kombucha-treated pigs are consistent with previous findings where fermented liquid strategies were studied for swine nutrition (Van Winsen et al., 2002; Canibe and Jensen, 2012). Fermented liquid feeds, characterized by low pH and elevated organic acid concentrations, have been shown to suppress *Enterobacteriaceae* populations in the gastrointestinal tract of pigs (Missotten et al., 2015; Xu et al., 2023). Canibe and Jensen (2012) reported significantly lower *Enterobacteriaceae* counts in growing pigs fed with fermented liquid feed diets as compared to non-fermented diets. Similarly, René L. Van Winsen et al. (2002) observed reductions in *Enterobacteriaceae* in pigs receiving grain-based fermented liquid diets inoculated with *Lactobacillus plantarum* as compared to control feed (non-fermented). These effects were attributed to the higher organic acid concentrations and lower pH of

fermented feeds, which reduced gastric pH, suppressed enteric pathogens, and supported the growth of beneficial microorganisms (René L Van Winsen et al. 2001; René L. Van Winsen et al. 2002). Kombucha shares several physical and chemical characteristics with fermented liquid feeds, including low pH, high organic acid content, and fermentation-derived bioactive compounds. This may explain the reductions observed in the present study for the kombucha groups. Choi et al. (2021) also observed a reduction in fecal coliform counts in post-weaned pigs after four weeks of kefir supplementation. Furthermore, Bhattacharya et al. (2016) identified multiple antimicrobial components in kombucha, including organic acids, bacteriocins, enzymes, and polyphenol derivatives, contributing to inhibitory activity against enteric bacteria. (AL-Kalifawi, 2014) also observed inhibitory activity of kombucha against gram-negative bacteria such as *Escherichia coli* and *Enterobacter cloacae.* Additionally, Battikh et al. (2013) reported strong antibacterial and antifungal effects of traditional kombucha and kombucha analogues produced from medicinal plant extracts, attributing these effects primarily to organic acids and other fermentation-derived bioactive compounds.

Fecal Microbial Diversity and Taxonomic Composition

Taxonomic composition and relative abundance of fecal samples at weeks 17, 21, and 23 in kombucha and isocaloric tea pigs are shown in Figure 3. Across all samples, fecal microbiota were dominated by *Treponema, Streptococcus, Prevotella, Bacteroides, Clostridium, Butyricicoccus, Fibrobacter, Oscillibacter, Ruminococcus, Lactobacillus, Pseudoflavonifractor, Methanobrevibacter, Blautia, and Eubacterium. Treponema, Prevotella,* and *Streptococcus* were consistently the most abundant genera across all groups and sampling times. The presence and dominance of these taxa are consistent with previous reports describing core members of the porcine fecal microbiota. Ramayo-Caldas et al. (2016) identified these genera across a large-

scale pig cohort (518 healthy pigs). Similarly, Solano-Aguilar et al. (2018) reported a comparable microbial profile in pigs fed fermented cocoa. *Streptococcus* relative abundance increased across all treatments by week 23. Similarly to our study, age- and growth stage–associated shifts in porcine gut microbiota composition have been previously reported, with *Treponema* and *Prevotella* predominating during earlier growth stages, whereas the finishing stage (>22 weeks of age) was associated with higher relative abundances of genera such as *Streptococcus, Lactobacillus,* and *Prevotella* (Luo et al., 2022). In our research, lean kombucha-treated pigs had an increase in *Lactobacillus* relative abundance by week 23. Members of *Lactobacillus* genus produce lactic acid and other metabolites, including short-chain fatty acids (SCFAs), which are known to play an important role in maintaining host gut health (Yang et al., 2014). Fermented feeding strategies have been associated with higher *Lactobacillus* relative abundance in pigs fed fermented liquid diets as compared to non-fermented controls (Bunte et al., 2020).

As observed in our study, host body type (lean vs obese) has been shown to influence these patterns. Pedersen et al. (2013) also reported higher *Lactobacillus* abundance in lean Ossabaw pigs as compared to obese group and associated these differences with a potential effect on intestinal permeability. Additionally, several genera, including *Eubacterium, Ruminococcus, Butyricicoccus,* and *Bacteroides*, were consistently detected across samples, independently of body type and treatments. These taxa have been widely reported in swine models across different ages and dietary conditions (Song et al., 2022; Zhao et al., 2025).

In our study, alpha diversity was assessed using Shannon index, which provides an estimate of microbial richness and evenness within each sample and serves as an indicator of community stability (Cassol et al., 2025). As shown in Figure 4, Shannon diversity was similar

between kombucha and isocaloric tea groups within each sampling week. Due to the exploratory nature of the study observed variations across weeks were interpreted as individual biological fluctuations rather than a significant association. Beta diversity analysis was assessed using Bray–Curtis dissimilarities and visualized by principal coordinates analysis (PCoA) to evaluate differences in microbial community composition between groups (Cassol et al., 2025). As shown in Figure 5 time and host body type had a greater influence on microbial community composition as compared to treatment (kombucha or isocaloric tea). Figure 5A showed overlapping microbial communities between kombucha and isocaloric tea group, with no significant difference between treatments. Conversely, in Figure 5B, body type-associated patterns (lean vs obese) were observed. At week 17 and 21, obese and lean pig samples were distributed in different regions of the ordination space. Our observations are consistent with previous studies (Pedersen et al., 2013; Panasevich et al., 2018), where different host body types showed diverse microbial community structure. In this study, by week 23, fecal microbiota in obese pigs increasingly overlapped with those from lean pigs, indicating greater similarity in overall community composition over time (Figure 5B). This pattern may reflect changes related to age as reported by Luo et al. (2022), or related to the transition of obese pigs from a Western-type diet to a standard diet at week 17.

Conclusions

This study offers an initial framework to understand the potential health benefits of sorghum kombucha consumption. A significant interaction was observed between kombucha consumption and the decrease of *Enterobacteriaceae* shedding in Ossabaw pig, independently of the host body type (lean vs obese). Fecal microbiota composition and diversity was instead influenced by host body type but not by treatment (kombucha vs isocaloric tea). Further research

incorporating longer feeding periods might elucidate the role of host body type and the influence on fecal microbiota when Ossabaw pigs are fed with alternative fermentative beverages such as sorghum kombucha.

Acknowledgments

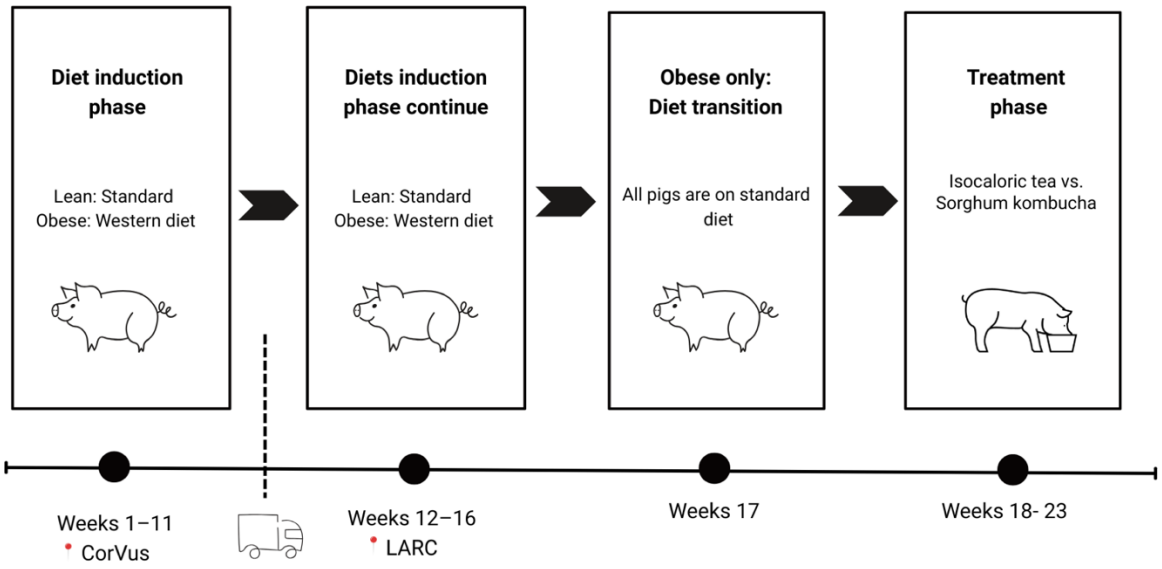
Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

Figures and Tables

Table 5 Effect of kombucha tea intervention and isocaloric tea on Enterobacteriaceae levels (log CFU/g) in lean and obese Ossabaw pigs over six weeks.

Week	Body Type	Kombucha tea	Isocaloric tea
17	Lean	5.78 ± 0.19^{bA}	6.60 ± 0.56^{aA}
	Obese	7.04 ± 0.67^{aB}	7.02 ± 0.28^{aA}
21	Lean	6.28 ± 0.23^{bA}	7.07 ± 0.59^{aA}
	Obese	6.44 ± 0.58^{aA}	6.95 ± 0.28^{aA}
23	Lean	6.25 ± 0.45^{aA}	6.70 ± 0.26^{aA}
	Obese	6.39 ± 0.42^{bA}	6.97 ± 0.15^{aA}

Different lowercase letters (a-b) within each row week indicate significant differences between treatments within the same body type and week. Upper case letters (A-B) denote differences between body type within the same treatment and week (A-B). (Sidak-adjusted pairwise comparisons, data shown as mean ± standard deviation; $p < 0.05$).



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1398 **Figure 2** Timeline of Experiments. CorVus (Crawfordsville, IN; AAALAC-accredited); LARC(
1399 Large Animal Research Center).

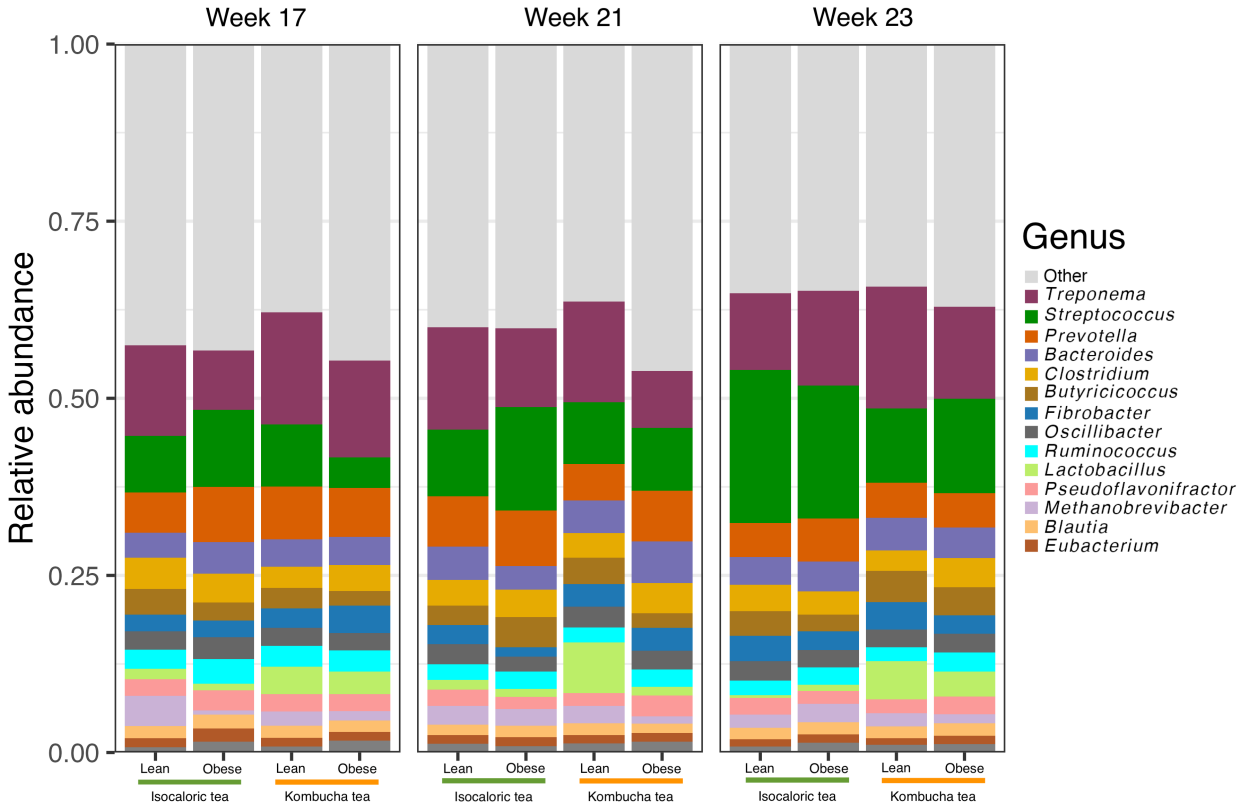

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Figure 3 Relative abundance of bacterial genera in fecal samples from Oassabaw pigs across treatments at weeks 17, 21, and 23.

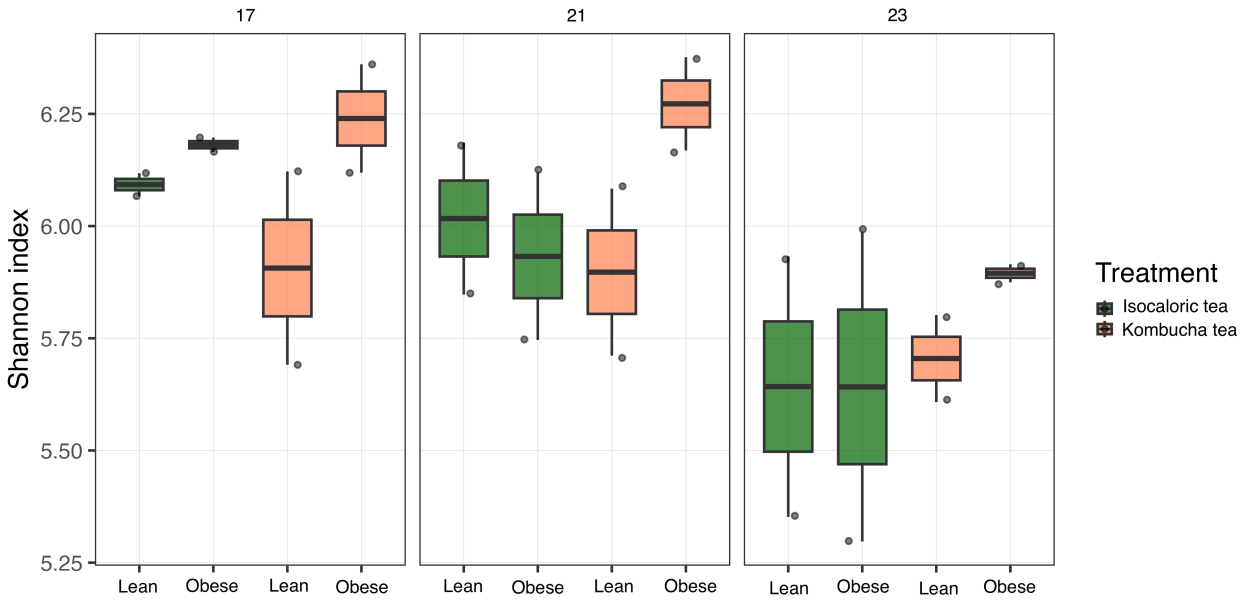
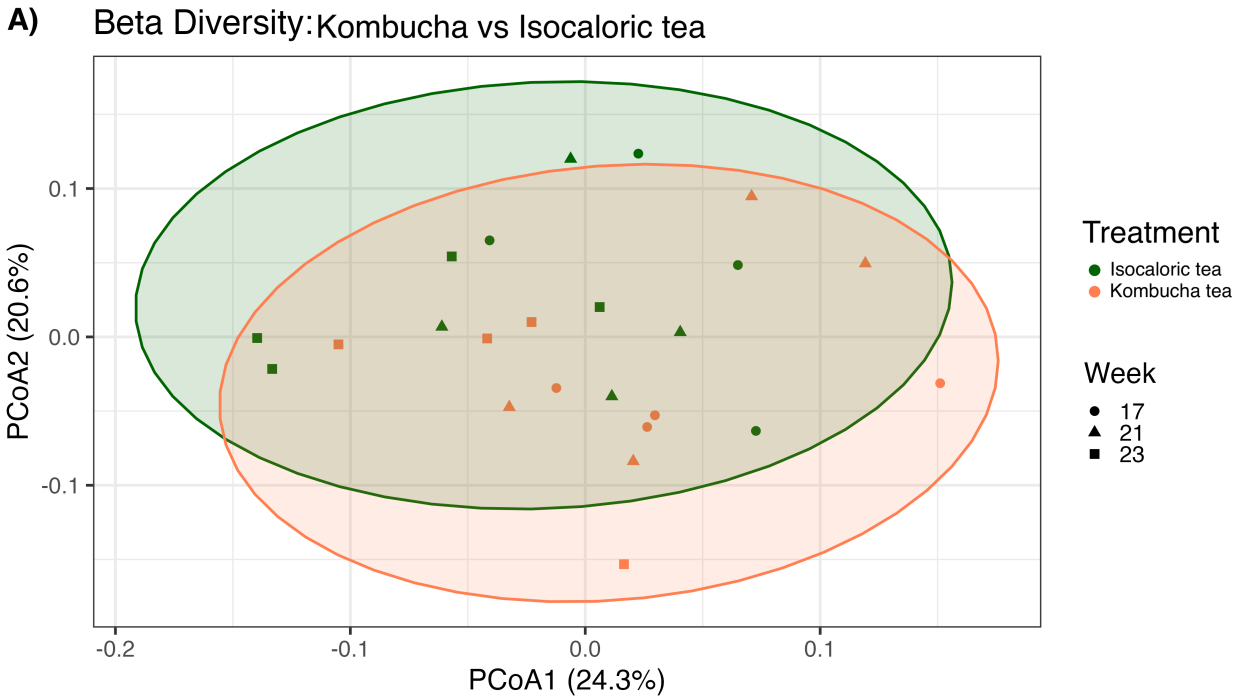


Figure 4 Alpha diversity of fecal samples from Ossabaw pigs across treatments and weeks (17, 21, 23



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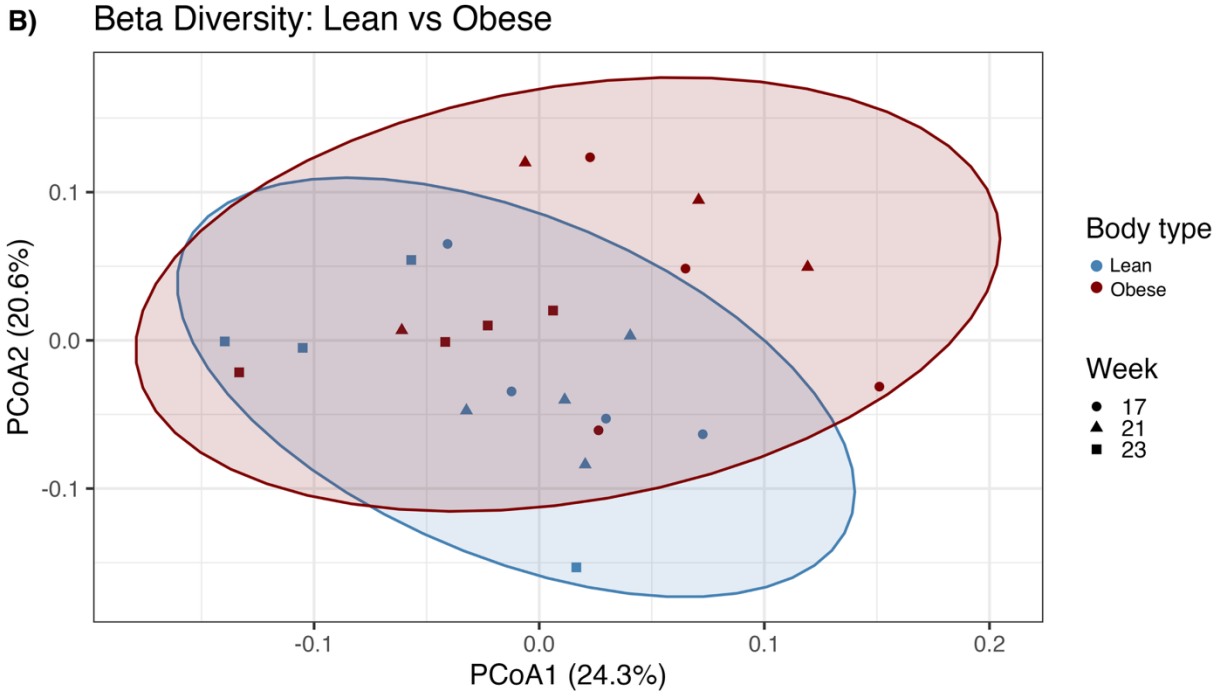

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1411 **Figure 5** Beta diversity of fecal microbiota from Ossabaw pigs based on Bray-Curtis
1412 Dissimilarities and visualized by principal coordinates analysis (PCoA). Points represent

1413 individual fecal samples. A) Microbial community structure by treatment across sampling weeks.
1414 B) Microbial community structure by body type across sampling weeks.
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Chapter 5 - Lactic Acid Bacteria Solid State Fermentation Improves Sorghum Grain Digestibility

Abstract

Sorghum is a gluten-free cereal valued for its resilience and agronomic performance; however, its use in human foods is limited by antinutritional factors and low protein digestibility. Solid-state fermentation (SSF) can improve sorghum nutritional quality by altering microbial metabolism and biochemical pathways. This study evaluated the effects of SSF on microbial growth, pH, phytic acid reduction, and in vitro protein digestibility of whole sorghum grains. Three whole-grain sorghum varieties (black, sumac, and white) were fermented at 30 °C for 72 h at either 30% or 50% moisture. *Pediococcus acidilactici* and *Leuconostoc mesenteroides* were used as starter cultures, either individually or combined (1:1), and microbial growth was monitored over time. pH and phytic acid were measured before and after fermentation. Selected treatments and their respective unfermented control were evaluated for in vitro protein digestibility (IVPD) using the INFOGEST method. Data were analyzed using mixed-effects models and analysis of variance ($\alpha = 0.05$). Microbial populations increased significantly in all tested samples ($p < 0.05$). Fermented treatments had lower pH and phytic acid content than their unfermented controls ($p < 0.05$). IVPD % increased significantly in fermented black and sumac sorghum ($p < 0.05$) after fermentation. These results highlight the use of SSF to enhance sorghum nutritional quality.

Introduction

Sorghum (*Sorghum bicolor* (L.) Moench) ranks as the fifth most produced grain globally, with an estimated production of 59.78 million metric tons between 2015 and 2024 (Hamaker et al., 2025; U.S. Department of Agriculture, Foreign Agricultural Service, 2025). This grain is a gluten-free cereal valued for its agronomic resilience (Santos D'Almeida et al., 2025). Due to its drought tolerance and suitability for marginal environments, sorghum is increasingly positioned as a climate-resilient crop (Meena et al., 2022).

Sorghum contains approximately 8–18% protein, 70–80% carbohydrates, 2–3% lipids, and 6-7 % dietary fiber, along with vitamins, minerals, and bioactive phytochemicals (Tanwar et al., 2023). Nevertheless, sorghum is often considered nutritionally limited due to its relatively low protein digestibility and reduced mineral bioavailability as compared with other cereals (De Morais Cardoso et al., 2017). These limitations are largely associated with the grain structural and compositional characteristics (Duressa et al., 2018). Kafirins, sorghum storage proteins, form extensive disulfide cross-links that reduce enzymatic accessibility during digestion (Duressa et al., 2018). In addition, interactions between proteins and phenolic compounds, together with the presence of antinutritional factors such as phytic acid, further limit nutrient utilization(Brouns, 2021; Duodu et al., 2003).

Several processing strategies have been evaluated to address these limitations, including physical methods, thermal treatments, fermentation and biochemical approaches such as germination and malting. (Atanda Usman, 2018; Bhattarai et al., 2025; Sruthi et al., 2021). Fermentation has been associated with reductions in phytic acid and improvements in protein digestibility (Ojha et al., 2018; Terefe et al., 2021). Rahman & Osman (2011) reported significant reductions in phytic acid during sorghum dough fermentation. While

Venkatasubbaiah & Konasur (2020) observed a decrease in phytic acid levels in fermented sorghum flour. Ogodo et al. (2019) reported that lactic acid fermentation of sorghum flour increased protein digestibility by more than 40% after 48 h, as compared to unfermented samples. These improvements were generally attributed to microbial acidification and to enzymatic activities that promote phytic acid hydrolysis and protein modification (Nkhata et al., 2018; Tsafrakidou et al., 2020). Solid-state fermentation (SSF), which involves microbial growth on solid substrates with limited free water, represents an attractive alternative for cereal processing due to its relatively low energy requirements (Álvarez et al., 2025; Feng et al., 2024). In SSF systems, moisture content is a critical factor regulating microbial growth, substrate porosity, oxygen diffusion, and overall metabolic activity (H. Chen, 2013). Both excessive and insufficient moisture levels can impair fermentation performance by altering matrix structure or limiting nutrient and enzyme diffusion, emphasizing the importance of optimizing moisture conditions (Sadh et al., 2018). Although SSF has been applied to improve the nutritional quality of several cereals and legumes (Feng et al., 2024), its application to whole sorghum grains remains underexplored. Most studies have focused on flour-based systems (Ogodo et al., 2019; Ojha et al., 2018) rather than on whole sorghum grains. Therefore, the objective of this study was to evaluate the effects of LAB-mediated solid-state fermentation of whole sorghum grains (black, sumac, and white) on lactic acid bacteria growth, pH reduction, phytic acid content, and in vitro protein digestibility.

Materials and Methods

Microbial strains

Pediococcus acidilactici (TN2) and *Leuconostoc mesenteroides* (TN1) were used as starter cultures in this study. Both strains were obtained from the culture collection of Dr. Faith Critzer currently at the University of Georgia (Athens, GA, USA). Bacterial strains were retrieved from frozen stocks (−80 °C), streaked onto de Man, Rogosa, and Sharpe (MRS) agar (Difco, Detroit, MI, USA), and incubated for 48 h at 35 °C for *P. acidilactici* or 30 °C for *L. mesenteroides*. A single isolated colony was transferred into 10 mL of MRS broth and incubated under the same conditions for 48 h to achieve a final level of 8 log CFU/mL.

Fermentable Substrate

Three commercially available whole-grain sorghum (*Sorghum bicolor* (L.) Moench) varieties were used as fermentation substrates. Black, sumac, and white grains were obtained from Nu Life Market LLC (Scott City, KS, USA). Black sorghum is characterized by a darkly pigmented pericarp and has been reported to contain elevated levels of phenolic compounds (Pfeiffer & Rooney, 2016). Sumac sorghum is defined by the presence of a red-brown pigmented testa layer associated with condensed tannins and low-molecular-weight phenolic compounds (Awika & Rooney, 2004; Scott et al., 2026). While white sorghum has white pericarp, and is a non-tannin type, generally exhibiting lower phenolic content than pigmented sorghum varieties (Tuinstra, 2008).

Solid State Fermentation (SSF)

SSF of whole sorghum grains was conducted as follows: for each fermentation batch, 100 g of whole sorghum grains were rinsed with deionized (DI) water, drained, transferred into a 1L beaker containing DI water (500 mL), and boiled for 5 min to reduce contamination and prevent

sprouting (Kim et al., 2022). Individual or combined cultures were added at 5% (v/w, dry basis). Target moisture levels of 30% and 50% were selected based on preliminary trials to represent low and intermediate conditions reported for solid-state fermentation systems (H. Chen, 2013; Yang et al., 2021). Moisture calculations were performed on a dry matter basis using the equation described by J. Chen & Zhu (2013):

$$W = DM\ (\frac{Mt}{1 - Mt})$$

where W is the total liquid added (g), DM is the dry matter (g), and M_t is the target moisture fraction. Flasks were covered with sterile coffee filters secured with rubber bands and incubated statically at 30 °C for 72 h. All fermentations were conducted in triplicate.

Lactic Acid Bacteria Enumeration

Microbial enumeration was performed at 2 h, 24 h, and 72 h of fermentation. At each sampling point, flasks were manually mixed with a sterile spatula, and 5 g of fermented matrix was aseptically transferred into sterile-filtered stomacher bags containing 45 mL of 0.1% peptone water (Bacto, Sparks, MD, USA). Samples were hand-massaged for 1 min, serially diluted in peptone water, plated in duplicate on Man, Rogosa, and Sharpe (MRS) agar (Difco, Detroit, MI, USA), and incubated at 32 °C for 48 h. All counts were expressed as log CFU/g of sample.

pH Measurement and Crude Protein Quantification

pH was measured before (unfermented control grains) and after fermentation (72 h) for all samples, following the AACC method 02-52.01. Briefly, 3.0 g of finely ground sorghum were suspended in 30 mL of deionized water (DI) (1:10, w/v), stirred for 15 min, and allowed to rest for 10 min before measurement. pH was recorded using a calibrated electronic pH meter

(SevenExcellence S400, Mettler Toledo, Columbus, OH, USA). All measurements were performed in triplicate.

Crude protein content was determined at 2 h, 24 h, and 72 h according to the AOAC 990.03 combustion method (LECO TruSpec CN, LECO Corp., MI), using a conversion factor of 6.25.

Phytic Acid Content

Phytic acid, a key antinutritional factor associated with reduced mineral bioaccessibility and protein digestibility (Gupta et al., 2013; Brouns, 2021) was quantified before (unfermented control grains) and at the end of the fermentation process (72 h) using the Megazyme K-PHYT enzymatic assay kit (Megazyme International, Bray, Ireland). Lyophilized samples (1 g) were extracted in 20 mL of 0.66 M HCl for 16 h and neutralized with 0.75 M NaOH following the manufacturer's instructions. Absorbance was measured at 655 nm using a UV–visible spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Results were expressed as mg/g dry matter.

Multi-criteria treatment selection framework

Due to the number of fermentation treatments (18) and the expensive nature of vitro protein digestion analyses, a multi-criteria selection framework was used to select treatments for in vitro protein digestion analysis. To allow comparison between variables with different scales, values were standardized using a z-score transformation before integration. A weighted composite index was calculated as:

$$Selection\ index = 0.75\ x\ (z - phytic\ acid\ reduction) + 0.25\ x\ (z - LAB\ counts)$$

Phytic acid reduction and LAB counts were considered in this framework, with phytic acid reduction given the highest weight due to its direct relevance to mineral bioavailability and

digestive enzymes (Brouns, 2021), compared with LAB counts, which reflected fermentation performance. Weights were derived using principal component analysis (PCA), and resulting rankings were compared using Spearman's rank correlation. The highest-ranked treatments (up to 6) were selected for subsequent in vitro protein digestibility analysis

In vitro protein digestibility (IVPD)

In vitro protein digestion was performed following the INFOGEST 2.0 standardized protocol (Brodkorb et al., 2019), consisting of sequential oral, gastric, and intestinal phases, with minor modifications. Simulated salivary fluid, simulated gastric fluid, and simulated intestinal fluid, were prepared according to Minekus et al., (2014) and prewarmed to 37 °C. Lyophilized samples (2 g) were reconstituted with 8 mL of sterile DI water, covered with aluminum foil, and stirred for 30 min at 37 °C. The oral phase was initiated by adding 6.4 mL of simulated salivary fluid, 40 µL of 0.3 M $CaCl_2$, and 1.56 mL of deionized water. The pH was adjusted to 7, followed by the addition of α-amylase (75 U/mL; Sigma-Aldrich, A3176). The mixture was incubated for 2 min at 37 °C with continuous stirring. For the gastric phase, the oral chyme was combined with 10.92 mL of simulated gastric fluid, 6 µL of 0.3 M $CaCl_2$, and 0.6 mL of DI water. The pH was adjusted to 3, after which pepsin (2000 U/mL; P6887) and gastric lipase (60 U/mL; L3126) were added. Gastric digestion was carried out for 120 min at 37 °C with continuous stirring. The intestinal phase was initiated by adding 11.0 mL of simulated intestinal fluid, 40 µL of 0.3 M $CaCl_2$, and 8.7 mL DI water. The pH was adjusted to 7, and pancreatin (100 U/mL; P7545) was added; digestion was performed for 120 min at 37 °C under continuous stirring. Aliquots were collected at the end of each digestion phase, and enzymatic activity was stopped by heating to 95 °C for 10 min. Digesta were centrifuged at 4500 × g for 10 min, and the

supernatant was used for soluble protein quantification using the bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL, USA) at 562 nm. All digestions were performed in triplicate.

IVPD (%) was calculated following Zhou et al., (2025):

$$IVPD\ (\%) = \left(\frac{P_s}{P}\right) \times 100$$

where P_s represents soluble protein content after digestion and P represents protein content before digestion.

Experimental Desing and Statistical analysis

Eighteen treatment combinations were evaluated in a completely randomized design (CRD) with a 3 × 3 ×2 factorial structure, comprising three grain varieties, three inoculum levels, and two moisture contents, with three replicates per combination, for a total of 54 experimental units. Response variables differed in their sampling structure: LAB counts, and crude protein content were measured at 2, 24, and 72 h, whereas pH, phytic acid, and IVPD% were measured before (unfermented grain) and after fermentation (72 h).

LAB (log CFU/g) and crude protein content were analyzed using linear mixed-effects models to account for repeated measurements over fermentation time. Fixed effects included variety, moisture, inoculum, time, and interactions, with replicates included as a random effect. Estimated marginal means were obtained, and pairwise comparisons were performed using Sidak-adjusted tests. Final pH and phytic acid were analyzed using a two-way ANOVA (variety × treatment), with treatment comprising the unfermented control and fermented treatments at 72 h. IVPD % was analyzed for the selected top-ranked treatments (see Section 2.6) using one-way ANOVA within each variety, followed by pairwise comparisons. All statistical analyses were performed in R (version 4.5) using the lme4 package for fitting linear mixed models (Bates et al.,

2015), and the emmeans package for estimated marginal means and comparisons (Lenth & Piaskowski, 2017). Statistical significance was set at $\alpha = 0.05$ for all tests.

Results and Discussion

Lactic Acid Bacteria Growth During Solid-State Fermentation

LAB are widely used in fermentation systems due to their ability to improve safety and enhance nutritional quality (Anumudu et al., 2024; Jeyakumar & Lawrence, 2022). However, successful application depends on whether substrate composition and processing conditions support strain-specific growth (Luo et al., 2025). In this study, LAB populations increase significantly across all samples over time ($p<0.05$; Table 6), confirming that the three sorghum varieties (black, sumac, white) supported microbial proliferation under the evaluated SSF conditions. Significant interactions were observed for variety × inoculum × time, and for moisture × inoculum × time ($p<0.05$), indicating that LAB growth depended on both grain variety and moisture levels. In black sorghum, samples inoculated with *P. acidilactici* at 30% moisture reached the highest population after 72 h (9.19 ± 0.37 log CFU/g), while samples inoculated with *L. mesenteroides* at 30% and 50% moisture exhibited lower counts (8.38 ± 0.42 and 8.39 ± 0.16 log CFU/g, respectively). In Sumac sorghum, at the end of the fermentation, the highest LAB population was observed in samples inoculated with *L. mesenteroides* at 50 % moisture (8.50 ± 0.17 log CFU/g), whereas the lowest counts were reported for samples inoculated with *P. acidilactici* at 50% (7.84 ± 0.10 log CFU/g). Conversely, in white sorghum, no significant differences were observed among samples by moisture % or inoculum type ($p > 0.05$) at the end of the fermentation.

Condensed tannins have been reported to influence the growth of LAB populations (Huang et al., 2024). Both black and sumac sorghums are phenolic-rich grains (Shen et al.,

2018). Sumac contains high levels of condensed tannins (proanthocyanidins), whereas black sorghum is a non-tannin variety characterized by high concentrations of 3-deoxyanthocyanidins (Awika & Rooney, 2004; Pfeiffer & Rooney, 2016). White sorghum, also non-tannin, generally exhibits lower total phenolic content than pigmented sorghum varieties (Kaufman et al., 2013).

Overall, the responses to phenolic composition were species specific. Similarly, Peng et al. (2018) observed reduced *Pediococcus* spp. and increased *Leuconostoc* spp. populations in tannin-rich ensiling systems. Similarly, Wang et al., (2022) observed decreased *Pediococcus spp.* populations during high-tannin silage fermentation. These findings are consistent with species-dependent responses to phenolic composition observed in our study where significantly higher *L. mesenteroides* counts in sumac sorghum and *P. acidilactici* counts in black sorghum were observed.

pH changes after 72 h of solid-state fermentation and crude protein content

Significant effects of variety, treatment (unfermented vs. fermented), and their interaction were observed in our study ($p < 0.05$). Initial pH values were measured as 6.32 ± 0.01 (unfermented black), 6.44 ± 0.14 (unfermented sumac), and 6.51 ± 0.02 (unfermented white). After 72 h, all treatments exhibited significantly lower pH as compared to the initial value ($p < 0.05$; Table 7), due to microbial organic acid production (J. Chen & Zhu, 2013). In black sorghum, treatments at 30% moisture consistently report lower pH values, as compared to the 50% moisture samples. Black sorghum inoculated with *P. acidilactici* at 50% moisture had the highest pH (5.78 ± 0.05; $p <0.05$) after 72 h. In sumac the lowest ($p<0.05$) pH values were observed for samples inoculated with *P. acidilactici* (5.10 ± 0.01) and *L. mesenteroides* (5.11 ± 0.02) at 30% moisture, as well as for the combined culture samples at 50% moisture (5.16 ± 0.03). In white sorghum, the lowest pH values were observed for the samples inoculated with *P.*

acidilactici (4.87 ± 0.01) and *L. mesenteroides* (4.91 ± 0.03), both at 30% moisture. Similar to black and sumac samples, in white sorghum the highest ($p<0.05$) pH value after 72 h was observed in samples inoculated with *P. acidilactici* at 50% of moisture (6.01 ± 0.01).

No significant effects of variety, moisture, inoculum, or time, nor any significant interactions among these factors, were detected for crude protein ($p > 0.05$). Mean ± standard deviation values are reported in Table A1.

Phytic acid changes during fermentation

Phytic acid degradation during cereal fermentation has been linked to changes in pH (Tsafrakidou et al., 2020), as pH below 6 enhances phytate solubility and microbial enzymatic hydrolysis (Coulibaly et al., 2010; Gupta et al., 2015). The decrease in phytic acid observed in our study is consistent with previous reports in cereal fermentation systems in which LAB activity promotes phytate hydrolysis (Fang et al., 2023). Venkatasubbaiah & Konasur, (2020) observed a phytic acid decrease in sorghum flour fermented with lactic acid bacteria after 27 h. Similarly, Rahman & Osman (2011) reported a decrease in phytic acid in sorghum dough after 24 h of fermentation. Similar findings have also been reported in SSF of wheat bran, where phytic acid reduction was observed alongside a decline in pH during lactic acid fermentation (Zhao et al., 2017). Reducing phytic acid can increase protein accessibility, as it has been shown to inhibit key digestive enzymes, including amylases, lipases, and proteases, thereby limiting macronutrient hydrolysis and overall nutrient bio accessibility (Zhang et al., 2025). Based on our statistical analysis, sorghum variety, treatment, and their interaction had a significant effect ($p < 0.05$) on phytic acid content during fermentation. As reported in Figure 6, initial phytic acid concentrations of unfermented grains were 15.13 ± 0.56 mg/g for black, 16.82 ± 0.31 mg/g for sumac, and 5.44 ± 0.56 mg/g for white sorghum. After 72 h, all fermented samples exhibited

significantly lower phytic acid levels as compared to their unfermented control grains ($p < 0.05$). In black sorghum, the greatest reductions ($p < 0.05$) were observed for samples at 30% moisture inoculated with *P. acidilactici* (6.60 ± 0.42 mg/g) and combined culture (6.07 ± 0.34 mg/g). While the highest phytic acid concentration was reported in 50% of moisture samples inoculated with *L. mesenteroides* (9.79 ± 0.22 mg/g). In Sumac sorghum, the lowest phytic acid values were observed in samples inoculated with *L. mesenteroides at 50% of moisture* (6.57 ± 0.39 mg/g) and the combined culture at 30% of moisture (6.41 ± 0.62 mg/g). The highest levels were reported for *P*. acidilactici (10.35 ± 0.57 mg/g) and the combined culture (9.63 ± 0.25 mg/g), both at 50% moisture ($p<0.05$). In white sorghum, the lowest values were observed at 50% moisture for samples inoculated with *P. acidilactici* (3.33 ± 0.27 mg/g) and combined culture (3.40 ± 0.16 mg/g). Higher phytic acid levels ($p<0.05$) after 72 h were reported at 30% of moisture for samples inoculated with *P. acidilactici* (4.48 ± 0.17 mg/g) and *L. mesenteroides* (4.35 ± 0.15 mg/g).

Treatment selection

Six treatments were selected for in vitro digestion analysis based on a multicriteria selection composite score index (Figure 7). The index integrated LAB counts and phytic acid reduction after 72 h of fermentation. The selected treatments were: black sorghum at 30% moisture inoculated with *P. acidilactici* and with the combined culture; sumac sorghum at 30% moisture inoculated with *P. acidilactici*, with *Leuconostoc mesenteroides*, and with the combined culture; sumac sorghum at 50% moisture inoculated with *L. mesenteroides*.

In vitro protein digestibility

Protein digestibility reflects the susceptibility of proteins to enzymatic proteolysis and is influenced by intrinsic protein structure and the presence of antinutritional factors (Sá et al.,

2020). In this study, fermentation significantly increased IVPD % across the evaluated samples compared with unfermented grains (Figure 8), demonstrating that solid-state fermentation of whole sorghum grains can enhance in vitro protein digestibility.

Improvements in sorghum protein digestibility following fermentation have been widely reported (Day & Morawicki, 2018). Ogodo et al., (2019) fermented sorghum flour with lactic acid bacteria and observed a significant increase in protein digestibility after 48 h of fermentation (>40%) as compared to unfermented flour. Similarly, Pranoto et al., (2013b) evaluated natural and *Lactobacillus*-mediated fermentation of sorghum flour and observed increases in digestibility (>47%) after 36 h of fermentation. The IVPD% values observed in our study (28–36%) were lower than those reported for flour-based sorghum fermentations above. These differences are likely due to structural distinctions between whole grains and milled matrices.

In our experiments, black sorghum, IVPD % increased from 15.6% in the unfermented grain to 35.1% in the samples inoculated with combined culture and 36.3% for the samples inoculated with *P. acidilactici* treatment, both at 30% moisture ($p<0.05$). In sumac sorghum, fermentation significantly increased IVPD % from 12.64% in the unfermented grain to a range of 28.1% - 36.4% across fermented samples ($p<0.05$). The highest values were observed in samples inoculated with *P. acidilactici* at 30% moisture (36.1%) and *L. mesenteroides* at 50% moisture (36.4%). Samples inoculated with the combined culture at 30% moisture content had the lowest IVPD% (28.1%).

Conclusion

This study demonstrates that LAB solid-state fermentation reduces phytic acid content and improves in vitro protein digestibility in whole sorghum grains. The magnitude of these improvements depended on sorghum variety (black, sumac, white), inoculum type (*P.acidilactic,*

L.mesenteroides or combined culture), and moisture level (30% or 50%), indicating that fermentation conditions should be tailored to the specific characteristics of each substrate. Further research evaluating changes in polyphenol composition during fermentation and their influence on microbial performance and enzymatic activity would support process optimization and provide a strategy to enhance the nutritional quality of whole-grain sorghum.

Acknowledgments

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Figures and tables

Tables and Figures

Table 6 Lactic acid bacteria enumeration during 72 h of solid-state fermentation across treatments.

Variety	Moisture	Inoculum	2 h	24 h	72 h
Black	30%	*P.acidilactici*	7.11 ± 0.32^{cA}	8.23 ± 0.23^{bA}	9.19 ± 0.37^{aA}
		L.mesenteroides	7.26 ± 0.13^{cA}	8.12 ± 0.29^{bA}	8.38 ± 0.42^{aB}
		Combined Culture	7.20 ± 0.02^{bA}	8.28 ± 0.08^{aA}	8.61 ± 0.19^{aAB}
	50%	*P.acidilactici*	7.23 ± 0.26^{bA}	8.45 ± 0.03^{aA}	8.90 ± 0.35^{aAB}
		L.mesenteroides	7.54 ± 0.45^{bA}	8.09 ± 0.24^{aA}	8.39 ± 0.16^{aB}
		Combined Culture	7.11 ± 0.13^{bA}	8.44 ± 0.03^{aA}	8.76 ± 0.26^{aAB}
Sumac	30%	*P.acidilactici*	7.07 ± 0.10^{cA}	7.80 ± 0.31^{bA}	8.43 ± 0.71^{aAB}
		L.mesenteroides	7.29 ± 0.12^{bA}	8.17 ± 0.16^{aA}	8.22 ± 0.15^{aAB}
		Combined Culture	7.24 ± 0.05^{bA}	7.95 ± 0.11^{aA}	8.15 ± 0.02^{aAB}
	50%	*P.acidilactici*	7.27 ± 0.18^{bA}	7.84 ± 0.10^{aA}	7.84 ± 0.10^{aB}
		L.mesenteroides	7.17 ± 0.19^{bA}	8.10 ± 0.21^{aA}	8.50 ± 0.17^{aA}
		Combined Culture	7.05 ± 0.30^{bA}	7.96 ± 0.25^{aA}	8.32 ± 0.04^{aAB}

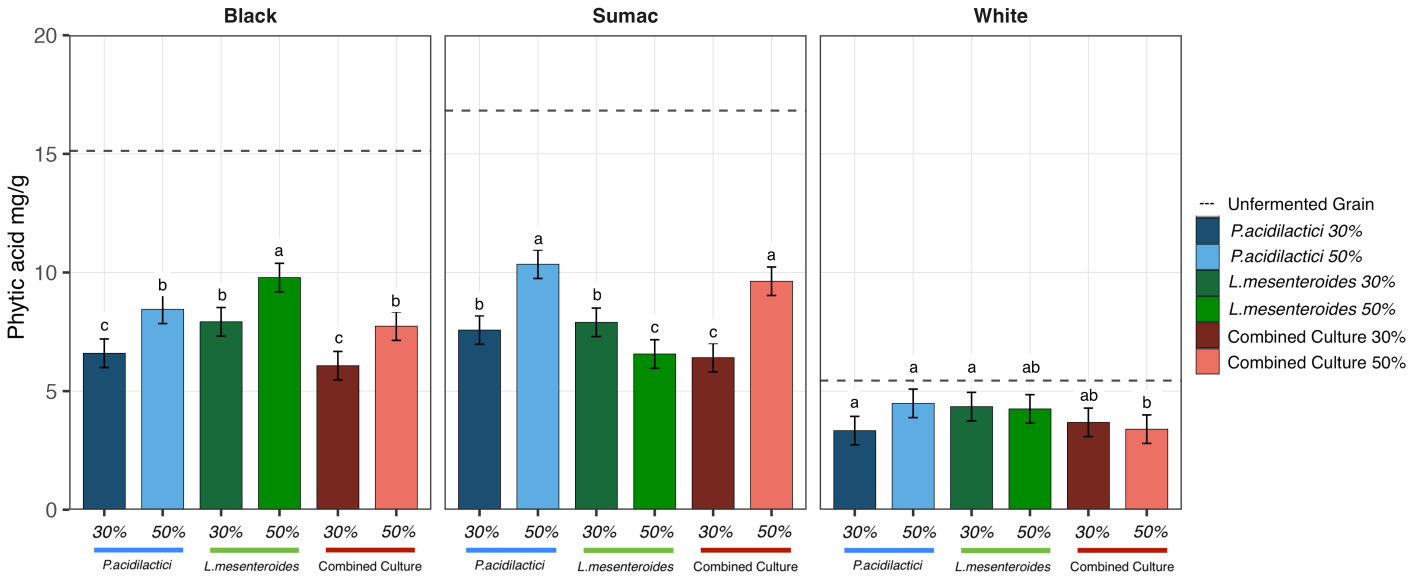
White	30%	*P.acidilactici*	7.34 ± 0.05^{bA}	8.04 ± 0.38^{aA}	8.71 ± 0.37^{aA}
		L.mesenteroides	7.19 ± 0.08^{bA}	7.96 ± 0.50^{aA}	8.18 ± 0.04^{aA}
		Combined Culture	7.33 ± 0.04^{bA}	8.16 ± 0.03^{aA}	8.31 ± 0.02^{aA}
	50%	*P.acidilactici*	7.24 ± 0.11^{bA}	8.12 ± 0.33^{aA}	8.37 ± 0.24^{aA}
		L.mesenteroides	7.25 ± 0.05^{bA}	8.30 ± 0.55^{aA}	8.45 ± 0.41^{aA}
		Combined Culture	7.23 ± 0.06^{bA}	8.15 ± 0.09^{aA}	8.41 ± 0.05^{aA}

Data are presented as mean ± standard deviation (n = 3). Within each row, different lowercase letters (a–c) indicate significant differences across time (2, 24, and 72 h). Within each column and sorghum varieties, uppercase letters (A–B) indicate significant differences among samples at the same time point ($p < 0.05$).

Table 7 pH values before and after 72 h of solid-state fermentation.

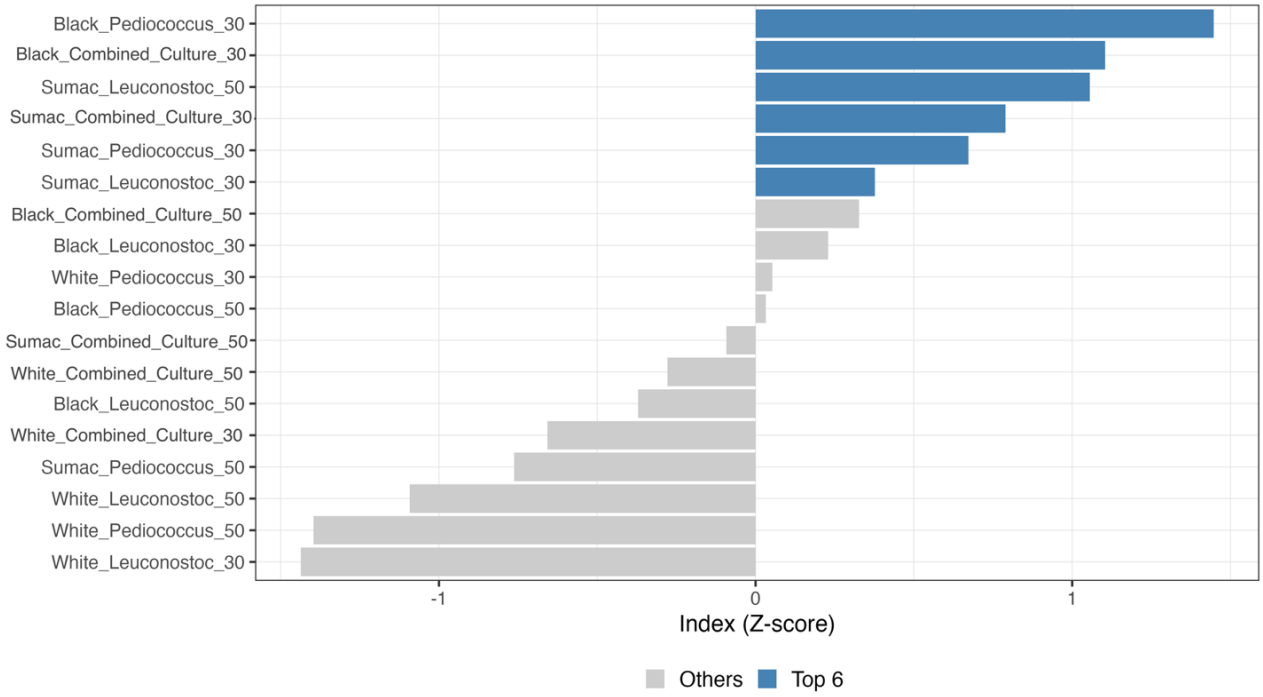
		Moisture/ Inoculum					
Grain Variety	Unfermented grain	*P.acidilactici*		*L.mesenteroides*		Combined Culture	
		30%	50%	30%	50%	30%	50%
Black	6.32 ± 0.01^{a}	4.86 ±0.04^{d}	5.78 ± 0.05 ^{b}	4.80 ± 0.03^{d}	5.22 ±0.06^{c}	4.82±0.04^{d}	5.12 ± 0.01^{c}
Sumac	6.44 ± 0.14^{a}	5.10 ±0.01^{e}	5.93 ± 0.02^{b}	5.11 ± 0.02 ^{e}	5.24±0.03^{cd}	5.27 ± 0.01^{c}	5.16 ± 0.03^{de}
White	6.51 ± 0.02^{a}	4.87 ±0.01^{e}	6.01 ± 0.01^{b}	4.91 ± 0.03^{e}	5.22 ±0.06^{c}	5.09 ± 0.02^{d}	5.24 ± 0.02 ^{c}

Data are presented as mean ± standard deviation (n = 3). Different lowercase letters within each row indicate significant differences among samples within the same sorghum variety ($p < 0.05$).



1866

1867 **Figure 6** Phytic acid concentration of sorghum grains before (unfermented grains) and after 72 h
1868 of solid-state fermentation. Bars represent the mean (n = 3) and error bars denote the 95%
1869 confidence intervals. Dash line represents unfermented grain. Within each grain variety, different
1870 lowercase letters indicate significant differences among treatments ($p < 0.05$).



1871

1872 **Figure 7** Composite treatment selection index based on phytic acid reduction and lactic acid
1873 bacteria counts at 72 h. Ranking consistency across weighting approaches was assessed using
1874 Spearman's rank correlation ($\rho = 0.98$). The six treatments selected for in vitro protein
1875 digestibility analysis are highlighted in blue.

1876

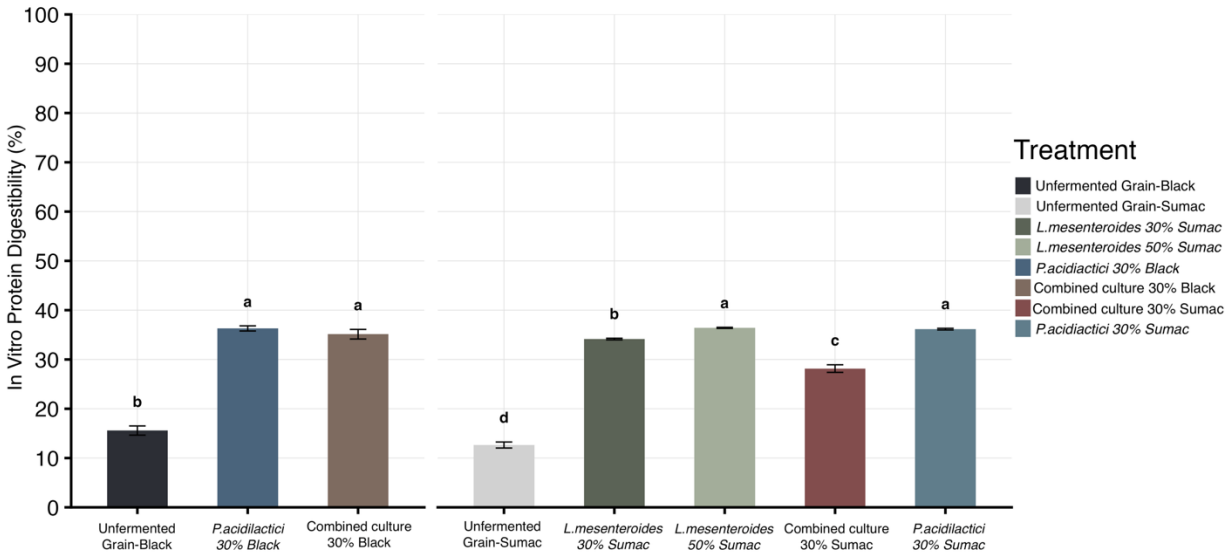


Figure 8 In vitro protein digestibility (IVPD) of selected fermented sorghum treatments and their corresponding unfermented grain. IVPD (%) was determined following in vitro digestion. Values represent mean ± standard deviation (n = 3). Within each sorghum variety (black or sumac), bars sharing different letters indicate statistically significant differences ($p < 0.05$).

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Chapter 6 - Conclusions

Sorghum is a resilient and drought-tolerant, nutritionally valuable cereal with significant potential for expanded use in human food systems. Nevertheless, its use remains limited by intrinsic characteristics that negatively affect nutrient availability and functionality. Fermentation represents a promising strategy to enhance sorghum value. As a biologically driven process, fermentation outcomes are shaped by interactions among microorganisms, substrate composition, processing conditions, and system-dependent effects.

Both submerged and solid-state fermentation (SSF) systems offer opportunities to expand sorghum applications. In submerged systems such as kombucha, sorghum was able to sustain the microbial consortium, maintaining profiles similar to those of the initial starter culture. In vivo evaluation in Ossabaw pigs model further suggested that sorghum kombucha may reduce *Enterobacteriaceae* shedding during consumption. However, fecal microbial composition was primarily influenced by host-related factors, and more controlled interventions might be required to better understand diet–microbiome interactions. Solid-state fermentation offers an alternative strategy that directly improves the nutritional quality of sorghum grain, reducing phytic acid and improving in vitro protein digestibility.

Across all studies, a consistent finding was the significant influence of sorghum variety and fermentation conditions on microbial activity and outcomes. Differences in grain composition influence fermentation dynamics and metabolites production, while

factors such as inoculum and moisture shaped the extent of nutritional improvement. These results emphasize that fermentation outcomes are determined by the combined effects of substrate characteristics and process conditions.

This research establishes a foundation for understanding sorghum fermentation and provides a framework for future work. Further studies should focus on elucidating polyphenol contents during fermentation and their role in microbial activity and nutrient bioaccessibility. Overall, fermentation is a versatile and effective approach to enhancing sorghum utilization. However, its success depends on a comprehensive understanding of the interactions between substrate properties, microbial communities, and processing conditions. Optimizing these interactions will be critical to fully realizing sorghum's potential as a high-value ingredient in global food systems.

Appendix A - Crude Protein Content

Table A1. Crude protein content across treatments and sampling points

Variety	Moisture	Inoculum	2 h	24 h	72 h
Black	30	*P.acidilactici*	7.35 ± 0.54	7.42 ± 0.57	7.33 ± 0.33
		L.mesenteroides	7.36 ± 0.08	7.60 ± 0.35	7.37 ± 0.27
		Combined Culture	6.62 ±0.6	7.68 ± 0.60	7.20 ± 0.48
	50	*P.acidilactici*	6.98 ± 0.41	6.33 ± 0.56	6.35 ± 0.08
		L.mesenteroides	7.16 ± 0.35	6.85 ± 0.46	6.59 ± 0.43
		Combined Culture	6.51 ± 0.78	6.26 ± 0.27	6.46 ± 0.45
Sumac	30	*P.acidilactici*	6.88 ± 0.67	6.28 ± 0.30	6.71 ± 0.44
		L.mesenteroides	6.90 ± 0.25	6.34 ± 0.46	7.81 ± 0.77
		Combined Culture	6.50 ± 0.52	6.85 ± 0.75	6.79 ± 0.68
	50	*P.acidilactici*	5.79 ± 0.42	5.58 ± 0.68	5.77 ± 0.09
		L.mesenteroides	5.83 ± 0.28	5.87 ± 0.69	4.83 ± 0.40
		Combined Culture	6.07 ± 0.66	5.67 ± 0.15	6.66 ± 0.60
White	30	*P.acidilactici*	7.04 ± 0.75	7.15 ± 0.76	7.55 ± 0.55
		L.mesenteroides	7.12 ± 0.22	7.08 ± 0.86	7.03 ± 0.85
		Combined Culture	6.75 ± 0.58	6.72 ± 0.44	6.22 ± 0.41
	50	*P.acidilactici*	6.11 ± 0.23	6.12 ± 0.24	6.05 ± 0.15
		L.mesenteroides	7.25 ± 1.04	6.40 ± 0.53	5.81 ± 0.23
		Combined Culture	6.18 ± 0.33	5.60 ± 0.17	6.31 ± 0.66

Data presented as mean ± standard deviation.